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Naphthalene- and Perylene-Based Linkers for the Stabilization of Hairpin Triplexes

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Abstract: Planar perylene- and naphthalene-based diimide linkers can be employed to tether the Watson-Crick and the Hoogsteen strands of a DNA triplex, thus providing conjugates capable of targeting single-stranded nucleic acids with the formation of hairpin triplexes. The planar linkers are designed to bridge the terminal base triplet of the three-stranded complex and provide base-stacking interactions with all three residues. Sixteen complexes have been prepared, eight with each linker, half with RNA (R) targets and half with DNA (D) targets. The conjugate sequences are composed of two strands of DNA, two of 2′-*O*-methyl RNA (M), or one of each. In comparison to similar complexes formed with a hexa(ethylene glycol) linker, the planar linkers enhance the T_M values for the complexes by as much as 28 °C with ΔG values indicating as much as 12.3 kcal/mol of stabilization relative to the simple glycol linker. All sixteen complexes have been characterized by T_M measurements and ΔG determinations. That π-stacking interactions are present between the linkers, and the nucleobases can be inferred from the quenching of the perylene fluorescence upon complex formation, and the observation of an absorbance vs temperature transition for the naphthalene-based linker at 383 nm and for the perylene-based linker monitored at 537 nm.

Introduction

Ligands have been used in numerous studies to target macromolecules such as proteins and nucleic acids. Site-specific binding by a ligand permits it to compete effectively with biological targets and thereby interfere with specific biological processes; nucleic acid therapeutics based upon an antisense or an antigene approach are examples of such targeted interference. A number of small-molecule ligands are known to bind to nucleic acid structures either by intercalation, groove binding, or other less well-characterized formats. In many cases such binding interactions function to over-stabilize duplex, triplex, or tetraplex structures, and such over-stabilization may contribute to an increased lifetime for the antisense or antigene complexes designed for gene-specific regulation of protein expression.

Conventional DNA triplexes are formed from a target duplex and a third single-stranded sequence.[1−3] Single-stranded sequences themselves can also be targeted by the triplex motif with sequences that can hybridize to the target by Watson−Crick base pairs and Hoogsteen base pairs.[4] In such complexes the two hybridizing arms are tethered either by a short nonbinding stretch of nucleotides,[5] or by a simple linker.[4,6]

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When both termini of the hybridizing sequence are connected, circular oligonucleotides result[7] that can very effectively bind single-stranded target sequences.[8] Enhanced stabilization of related complexes also results when two independently hybridizing sequences are tethered by a short linker.[3−5]

Enhanced triplex stabilization can also be achieved through additional binding interactions from a ligand that is covalently tethered to one of the macromolecular members of the DNA triplex.[9−12] Such covalent attachment usually results in significant proximity effects and provides, in part, entropic-based stabilization of the complex. A variety of ligands have been tethered to nucleic acids, and these include intercalators[9,13−15] and groove-binding agents,[10,11,16] as well as metal complexes.[17−19] Although the nature of the linker tethering the ligand to the nucleic acid sequence varies considerably, the site of attachment is usually one of the two nucleic acid termini, a nucleobase residue, or the backbone of the oligomer.

Previous studies have shown that naphthalene diimide chromophores function as effective intercalators[20,21] even polyintercalators for DNA. For example, L-lysine has been derivatized at it's ϵ-amino group, by attachment of a 1,8-naphthalimide moiety; this conjugate functions as a site specific DNA-cleaving agent.[22,23] Naphthalene diimide derivatives have additionally been studied for properties related to their redox activity.[24,25] A 1,4,5,8-naphthalene tetracarboxylic diimide chromophore attached in a head-to-tail arrangement by peptide linkers was suggested to be fully intercalated into DNA through the major groove.[26] Binding studies of a *N*-[3-(dimethylamino)-propylamine]-1,8-naphthalene dicarboxylic imide and *N*,*N*′-bis-[3,3′-(dimethylamino)propylamine]-naphthalene-1,4,5,8-tetracarboxylic diimide indicated that both of these compounds intercalated into a DNA duplex and preferred to intercalate into steps containing at least one G:C base pair.[27]

Earlier work with perylene derivatives has largely concentrated on spectral and fluorescence characterizations of the molecule.[28−30] Spectral characteristics of the naphthalene and perylene chromophores have been compared.[31,32] Red pigmentary polyimides have been synthesized from *N*,*N*′-diamino-3,4,9,10-perylenetetracarboxylic acid bisimide.[33] The *N*,*N*′-bis-[3,3′-(dimethylamino)propylamine]-3,4,9,10-perylene tetracarboxylic diimide (a dicationic dye) has been used for precipitation and quantitation of DNA,[34] and recently a related perylene tetracarboxylic diimide-based ligand was shown to bind G-tetraplex and to inhibit human telomerase.[35]

In preliminary communications we have reported that tethered derivatives of naphthalene and perylene diimides can function to stabilize DNA duplexes[36] and triplexes.[36−38] In the present work we report on the synthesis and stability of sixteen different hairpin triplexes containing either tethered naphthalene or perylene conjugates, as well as the fluorescence characteristics of perylene-based DNA conjugates.

Experimental Section

NMR spectra were determined on Varian spectrophotometers. Mass spectra were obtained using FAB ionization from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, Urbana, IL. MALDI-TOF mass spectral analyses of the oligonucleotides were performed by Dr. D. J. Fu, Sequenome, San Diego, CA. Rotary evaporations were performed under reduced pressure with Buchi systems. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} precoated on aluminum sheets (EM Separations Technology), in CH_3OH:CH_2Cl_2 (1:19) unless otherwise specified. All phosphitylation reactions were monitored with aluminum oxide IBF TLC sheets from JT Baker Inc. Flash column chromatography was performed on Silica Gel 60, Particle size 0.040−0.063 (EM Separations Technology), using a CH_3OH gradient in CH_2Cl_2 unless otherwise specified. CH_2Cl_2 and diisopropyl ethylamine were dried by heating over calcium hydride under reflux for several hours followed by distillation. Other anhydrous solvents and starting materials were purchased from Aldrich Chemical Co. and used without further purification unless otherwise specified. UV scans and absorbances were obtained using a Beckman DU 640 spectrophotometer. Oligodeoxynucleotides were synthesized on an Applied Biosystems 381A DNA Synthesizer. 2′-Deoxynucleotide phosphoramidites and 3′-terminal nucleoside-controlled pore glass support (CPG) were purchased from Glenn Research (Sterling, VA). The hexaethylene glycol linker, prepared as a 4,4′-dimethoxytrityl β-cyanoethyl phosphoramidite derivative was synthesized as described elsewhere.[36] High performance liquid chromatography (HPLC) was performed on a Beckman HPLC system using C-18 reversed-phase columns (ODS-Hypersil, 5 mm particle size, 120A pore) with detection at 260 nm. Oligonucleotides were desalted with Econo-Pac 10DG

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disposable chromatography Columns (Bio Rad, Hercules, CA). T_M measurements were performed on an AVIV Spectrophotometer, model 14DS UV/vis.

***N,N′*-Bis 2-(2-hydroxyethoxy)ethyl-1,4,5,8-naphthalenetetracarboxylic diimide (1).** To 1.0 g (3.7 mmol) 1,4,5,8-naphthalenetetracarboxylic dianhydride suspended in anhydrous pyridine (25 mL) was added 1.6 g (15 mmol) 2-(2-aminoethoxy)ethanol and 1.0 g (4.6 mmol) zinc acetate dihydrate. The mixture was refluxed overnight. TLC analysis indicated the absence of starting material and the presence of a new product. Pyridine was removed in vacuo. The residue was suspended in dichloromethane, and a small amount of methanol was added until the solids dissolved. Silica gel was added to the solution, and the solvents were removed in vacuo. The silica-absorbed material was poured onto a short column of silica gel dissolved in dichloromethane and eluted with 0−15% methanol. The fastest moving band corresponded to the diimide product, and 1.16 g (2.6 mmol) of **1** was obtained (70% yield). R_f: 0.14. ^{1}H NMR (DMSO-d_6) $\delta = 3.67$ (m, 8H, $-CH_2-$), 4.22 (t, 4H, $-$CH2$-$) 4.56 (t, 4H, $-CH_2$), 8.60 (s,4H of Ar H) ppm. ^{13}C NMR (DMSO-d_6) δ 49.4, 69.3, 81.2, 134.8, 135.1, 138.9, 171.5 ppm. UV (dichloromethane:methanol, 9/1): λ_{max} 377, 359, 241 nm. HRMS: calcd for $C_{22}H_{22}N_2O_8$ ($M + H^+$) 443.1454, found 443.1454.

***N*-2-[(2-*O*-4,4′-dimethoxytritylethoxy)ethyl]-*N*′-2-(2-hydroxyethoxy)-ethyl- 1,4,5,8-naphthalenetetracarboxylic diimide (2).** To 1.1 g (2.5 mmol) of *N,N*′-bis-2-(2-hydroxyethoxy)ethyl-1,4,5,8-naphthalenetetracarboxylic diimide **(1)** dissolved in anhydrous pyridine (25 mL) was added 0.89 g (2.6 mmol) 4,4′-dimethoxytrityl chloride. The reaction mixture was stirred for 3 h. TLC analysis at this time indicated the disappearance of **1** and the presence of two products. The reaction was stopped by adding several drops of methanol followed by the removal of solvents in vacuo. The mixture was dissolved in dichloromethane (50 mL) and was washed with an aqueous solution of saturated sodium bicarbonate (2 × 50 mL). The organic layer was dried over anhydrous sodium sulfate. The mixture was then vacuum filtered, and the filter was washed with dichloromethane. The mono(dimethoxytrityl) product was purified by flash chromatography using 1% triethylamine/dichloromethane and a methanol gradient. After evaporation of appropriate fractions, this material was precipitated into cold hexane to remove residual triethylamine. The precipitate was filtered and washed with cold hexane. The product was rinsed from the filter with dichloromethane and was concentrated to a yellow foam to yield 0.61 g (0.82 mmol) of **2** (32% yield). $R_f = 0.37$. A faster moving compound was also obtained, and it appeared to be the bis(dimethoxytrityl) product. $R_f = 0.71$. ^{1}H NMR ($CDCl_3$) $\delta = 3.18$ (t, 2H, $-CH_2-$), 3.66 (m, 6H, $-CH_2-$), 3.75 (s, 6H, OCH_3), 3.88 (t, 4H, $-CH_2-$), 4.49 (m, 4H, $-CH_2-$), 6.69 (d, 4H, Ar H), 7.1−7.4 (m, 9H, Ar H), 8.7 (s, 4H, Ar H) ppm. ^{13}C NMR ($CDCl_3$) δ 48.5, 48.6, 63.8, 70.4, 71.6, 76.5, 76.8, 79.1, 80.9, 94.4, 121.5, 135.0, 135.2, 135.2, 135.3, 136.3, 136.8, 138.6, 139.7, 144.8, 153.4, 166.8, 171.5, 171.8 ppm. UV (dichloromethane: methanol, 9/1): λ_{max} 379, 359, 241 nm. HRMS: calcd for $C_{43}H_{40}N_2O_{10}$ ($M + H^+$) 744.2683, found 744.2682.

***N*-2-[(2-*O*-4,4′-dimethoxytritylethoxy)ethyl]-*N*′-2-{[2-*O*-(2-cyanoethyl diisopropyl phosphino)ethoxy]ethyl}-1,4,5,8-naphthalenetetracarboxylic diimide (3).** To 30 mg (0.040 mmol) of *N*-2-[(2-*O*-4,4′-dimethoxytritylethoxy)ethyl]-*N*′-2-(2-hydroxyethoxy)ethyl-1,4,5,8-naphthalenetetracarboxylic diimide **(2)** dissolved in anhydrous dichloromethane (1 mL at 0 °C) was added 0.036 mL (0.20 mmol) diisopropylethylamine followed by 0.018 mL (0.080 mmol) 2-cyanoethyl diisopropylchlorophosphoramidite. The reaction was slowly brought to 25 °C and stirred for 30 min. TLC analysis indicated the absence of **2** and the presence of a new product. The reaction was stopped with 2−3 drops of methanol, and the phosphitylated product was precipitated in cold hexane (0 °C). The product was filtered, washed with cold hexane, and rinsed from the filter with dichloromethane. The product was concentrated to yield 0.026 g (0.028 mmol) of **3** as a yellow foam (70% yield). $R_f = 0.90$. ^{31}P NMR ($CDCl_3$, H_3PO_4 external reference) 148 ppm.

2-(2-*O*-*tert*-Butyldimethylsilylethoxy)ethylamine (4). To 5.0 g (48 mmol) of 2-(2-aminoethoxy)ethanol dissolved in anhydrous pyridine (50 mL) was added 7.2 g (48 mmol) *tert*-butyldimethylsilyl chloride and 4.9 g (72 mmol) of imidazole. The reaction mixture was stirred overnight. TLC analysis indicated the absence of starting material and formation of new products. Pyridine was removed in vacuo. The mixture was dissolved in dichloromethane (50 mL) and was washed with an aqueous solution of saturated sodium bicarbonate (2 × 50 mL). The organic layer was dried over anhydrous sodium sulfate. The mixture was then vacuum filtered, and the filter was washed with dichloromethane. The product was purified by flash chromatography to yield 3.5 g (16 mmol) of **4** as a brown oil (36% yield). R_f ($MeOH:CH_2Cl_2$, 1:9) = 0.80. ^{1}H NMR ($CDCl_3$): $\delta = 0.00$ (s, 6H, $SiCH_3$), 0.83 (s, 9H, $-CH_3$), 2.77 (m, 2H, $-CH_2-$), 3.44 (t, 2H, $-CH_2-$), 3.70 (m, 4H, $-CH_2-$) ppm. ^{13}C NMR (CDCl3): $\delta = 3.3$, 27.0, 34.4, 34.5, 50.4, 71.3, 81.0, 81.8 ppm. HRMS: calcd for $C_{10}H_{25}NO_2Si$ ($M + H^+$) 220.1733, found 220.1734.

***N,N′*-Bis-2-[(2-*O*-*tert*-butyldimethylsilylethoxy)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (5).** To 2.0 g (5.1 mmol) perylenetetracarboxylicdianhydride suspended in anhydrous pyridine (25 mL) was added 3.4 g (15 mmol) 2-(2-*O*-*tert*-butyldimethylsilylethoxy)-ethylamine **(4)** and 1.1 g (5.1 mmol) zinc acetate dihydrate. The reaction mixture was refluxed overnight. TLC analysis indicated the absence of starting material and the formation of products. Pyridine was removed in vacuo. The mixture was suspended in chloroform and loaded onto a large silica column. The product was purified by flash chromatography to yield 3.4 g (4.4 mmol) of **5** as a dark red solid (86% yield). R_f (methanol:dichloromethane, 1:9) = 0.90. ^{1}H NMR ($CDCl_3$): $\delta = 0.00$ (s, 12H, $SiCH_3$), 0.83 (s, 18H, $-CH_3$), 3.61 (t, 4H, $-CH_2-$), 3.68 (t, 4H, $-CH_2-$), 3.83 (t, 4H, $-CH_2-$), 4.44 (t, 4H, $-CH_2-$), 8.60 (q, 8H of Ar H) ppm. ^{13}C NMR ($CDCl_3$): $\delta = -4.2$, 19.7, 27.0, 40.5, 63.8, 69.0, 73.3, 123.6, 123.9, 126.8, 129.0, 131.8, 134.7, 163.9 ppm. UV (chloroform): λ_{max} 524, 489, 457, 265, 243. HRMS: calcd for $C_{44}H_{54}N_2O_8Si_2$ ($M + H^+$) 795.3490, found 795.3500.

***N*-2-[(2-*O*-*tert*-Butyldimethylsilylethoxy)ethyl]-*N*′-2-(2-hydroxyethoxy)ethyl-3,4,9,10,-perylenetetracarboxylic diimide (6).** To 3.4 g (4.2 mmol) *N,N*′-bis-2-[(2-*O*-*tert*-butyldimethylsilylethoxy)ethyl]-3,4,9,10-perylenetetracarboxylic diimide **(5)** dissolved in anhydrous pyridine (50 mL) was added HF/pyridine (70%) until approximately half of the starting material had lost one silyl-protecting group as monitored by TLC analysis (loss of both silyl-protecting groups resulted in an insoluble precipitate). Pyridine was removed in vacuo. The mixture was resuspended in chloroform and was purified by flash chromatography to yield 0.69 g (1.0 mmol) of **6** as a dark red solid (24% yield). R_f (methanol:dichloromethane, 1:9) = 0.43. ^{1}H NMR ($CDCl_3$): $\delta = 0.00$ (s, 6H, $SiCH_3$), 0.83 (s, 9H, $-CH_3$), 3.60−3.80 (m, 8H, $-CH_2-$), 3.90 (m, 4H, $-CH_2-$), 4.44 (m, 4H, $-CH_2-$), 8.0−9.0 (m, 8H of Ar H) ppm. UV (chloroform): λ_{max} 525, 489, 458, 263, 241. HRMS: calcd for $C_{38}H_{40}N_2O_8Si$ ($M + H^+$), 681.2632, found 681.2634.

***N*-2-[(2-*O*-4,4′-Dimethoxytritylethoxy)ethyl]-*N*′-2-[(2-*O*-*tert*-butyldimethyl-silylethoxy)ethyl]-3,4,9,10,-perylenetetracarboxylic diimide (7).** To 0.67 g (1.1 mmol) *N*-2-[(2-*O*-*tert*-butyldimethylsilylethoxy)-ethyl]-*N*′-2-(2-hydroxyethoxy)ethyl-3,4,9,10,-perylenetetracarboxylic diimide **(6)** dissolved in anhydrous pyridine (10 mL) was added 0.58 g (1.7 mmol) 4, 4′-dimethoxytrityl cholride. The reaction was stirred for 3 h. TLC analysis indicated the absence of starting material and the formation of a new product. The reaction was stopped with 1 mL of methanol. The solvents were removed in vacuo. The mixture was dissolved in chloroform to which 2 drops of triethylamine had been added. The mixture was purified by flash chromatography in 1% triethylamine/chloroform with a methanol gradient to yield 0.75 g (0.76 mmol) of **7** as a dark red solid (69% yield). R_f (methanol:dichloromethane, 1:9) = 0.87. ^{1}H NMR ($CDCl_3$): $\delta = 0.00$ (s, 6H, $SiCH_3$), 0.83 (s, 9H, $-CH_3$), 3.60−3.80 (m, 8H, $-CH_2-$), 3.80 (s, 6H, OCH_3), 3.90 (m, 4H, $-CH_2-$), 4.44 (m, 4H, $-CH_2-$), 6.72 (d, 2H, ArH), 6.83 (d, 2H, ArH), 7.1−7.4 (m, 9H, ArH), 8.25 (m, 4H, Ar H), 8.45 (m, 4H, Ar H) ppm. ^{13}C NMR ($CDCl_3$): $\delta = -4.1$, 19.7, 27.0, 40.7, 56.2, 56.3, 63.9, 64.3, 69.0, 69.3, 71.5, 73.3, 86.5, 86.8, 114.0, 114.2, 123.5, 123.5, 123.8, 123.8, 127.6, 128.7, 128.8, 128.9, 129.3, 130.2, 131.1, 131.7, 131.7, 134.5, 137.4, 137.4, 146.1, 159.3, 163.9 ppm. UV (chloroform): λ_{max} 525, 489, 458, 263, 243. MS: calcd for $C_{38}H_{39}N_2O_8$-Si (M^+), 982, found 619 (M^+ − DMT−OCH_2CH_2O-), 807 (M^+ − *t*BDMS−OCH_2CH_2O-), 444 (M^+ − DMT−OCH_2CH_2O and − *t*BDMS−OCH_2CH_2O-), 303 (DMT).

***N*-2-[(2-*O*-4,4′-Dimethoxytritylethoxy)ethyl]-*N*′-2-(2-hydroxy-**

ethoxy)ethyl-3,4,9,10,-perylenetetracarboxylic diimide (8). To 0.75 g (0.82 mmol) *N*-2-[(2-*O*-4,4′-dimethoxytritylethoxy)ethyl]-*N*′-2-[(2-*O*-*tert*-butyldimethylsilylethoxy)ethyl] 3,4,9,10,-perylenetetracarboxylic diimide **(7)** dissolved in anhydrous pyridine (20 mL) was titrated HF/pyridine (70%) until the starting material had been transformed as monitored by TLC analysis. Pyridine was removed in vacuo. The mixture was resuspended in chloroform and was purified by flash chromatography in in 1% triethylamine/chloroform with a methanol gradient to yield 0.36 g (0.41 mmol) of **8** as a dark red solid (50% yield). R_f (methanol:dichloromethane, 1:19) = 0.25. ^{1}H NMR ($CDCl_3$): δ = 3.10 (m, 2H, CH_2) 3.19 (t, 2H, CH_2) 3.68 (m, 4H, $-CH_2-$), 3.78 (s, 6H, OCH_3), 3.92 (t, 4H, $-CH_2-$), 4.50 (m, 4H, $-CH_2-$), 6.72 (d, 2H, ArH), 6.83 (d, 4H, ArH), 7.1−7.4 (m, 9H, ArH), 8.45 (m, 4H of Ar H), 8.60 (m, 4H of Ar H) ppm. ^{13}C NMR ($CDCl_3$): δ = 40.7, 40.9, 56.2, 63.0, 64.3, 69.3, 69.4, 71.5, 73.5, 86.8, 123.5, 123.6, 123.7, 123.8, 126.3, 127.6, 128.7, 129.3, 129.6, 129.7, 131.1, 131.8, 131.9, 134.4, 134.74 137.4, 146.0, 159.3, 163.9, 164.3 ppm. UV (chloroform): λ_{max} 525, 489, 458, 263, 243. HRMS: calcd for $C_{53}H_{44}N_2O_{10}$ ($M + H^+$) 869.3074, found 869.3071.

***N*-2-[(2-*O*-4,4′-Dimethoxytritylethoxy)ethyl]-*N*′-2-{[2-*O*-(2-cyanoethyl diisopropylchlorophosphino)ethoxy]ethyl}-3,4,9,10,-perylenetetracarboxylic diimide (9).** To 40 mg (0.055 mmol) *N*-2-[(2-*O*-4,4′-dimethoxytritylethoxy)ethyl]-*N*′-2-(2-hydroxyethoxy)ethyl-3,4,9,10-perylenetetracarboxylic diimide **8** dissolved in anhydrous dichloromethane (1 mL at 0 °C) was added 0.048 mL (0.28 mmol) diisopropylethylamine followed by 0.025 mL (0.11 mmol) 2-cyanoethyl diisopropylchlorophosphoramidite. The reaction was slowly brought to 25 °C and stirred for 30 min. TLC analysis indicated the absence of starting material and the presence of a new product. The reaction was stopped with 2−3 drops of methanol, and the phosphitylated product was precipitated into cold hexane (0 °C). The product was filtered, washed with cold hexane and rinsed from the filter with dichloromethane. The product was concentrated to yield 0.035 g (0.036 mmol) of **9** as a red solid (65% yield). R_f = 0.67. ^{31}P NMR ($CDCl_3$, H_3PO_4 external reference) 149 ppm.

Synthesis of Oligonucleotides Containing Linkers. DNA sequences were assembled using standard phosphoramidite protocols. The hexaethylene glycol and naphthalene linkers were incorporated on the DNA synthesizer by increasing the coupling time of the corresponding phosphoramidite from 30 s to 10 min. Oligonucleotides containing the perylene linker were assembled on the synthesizer up to the site of the linker. The final 4,4′-dimethoxytrityl (DMT)-protecting group was removed from the 5′-hydroxyl of the base 3′ to the linker and at this point the synthesis was interrupted. The CPG containing the oligonucleotide was removed from the synthesizer, dried in vacuo, and poured into a small flask. To the CPG was added 30 μmol of the perylene linker phosphoramidite (**9**) dissolved in 0.50 mL of anhydrous dichloromethane and 0.50 mL of 0.5 M tetrazole in acetonitrile. The reaction was mixed in an argon atmosphere for 1 h. The CPG was then filtered and rinsed with dichloromethane to remove unincorporated phosphoramidite. The CPG was replaced in the column which was reattached to the synthesizer and the synthesis was resumed at the capping step. This procedure was necessary owing to the moderate solubility characteristics of perylene derivative in acetonitrile. The base 5′ to the perylene linker was dissolved in anhydrous dichloromethane and incorporated by increasing the coupling time from 30 s to 10 min. The remaining bases were added on by standard methods with phosphoramidites dissolved in acetonitrile.

The terminal DMT groups were removed from the oligonucleotides containing the naphthalene and perylene linkers. The sequences were deprotected in concentrated methanolic ammonia at 55 °C overnight (perylene-based conjugates were typically deprotected in concentrated aqueous ammonia at 55 °C overnight). The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis using 20% polyacrylamide/1% bisacrylamide/7 M urea gels in 89 mM tris-borate buffer containing 2 mM Na_2EDTA, pH 8 (1× TBE buffer). The bands were visualized by UV shadowing (and by the obvious colored properties of the linkers) and excised. The pure oligonucleotides were electroeluted in 0.5× TBE buffer using a Schleicher and Schuell (Keene, NH) Elutrap. In contrast, after deprotection in concentrated aqueous ammonia at 55 °C overnight, the 5′-terminal DMT group was not cleaved from oligonucleotides containing the hexaethylene glycol linker. These oligonucleotides were purified by HPLC in 50 mM triethylammonium acetate buffer (pH 7) with a gradient of acetonitrile. The DMT group was then removed by treatment with acetic acid for 30 min. All oligonucleotides were desalted on columns of Sephadex G-10 prior to use.

***T*$_M$ Values.** T_M values were obtained for duplexes or complexes containing a 1:1 mixture of oligonucleotide-linker-oligonucleotide and single stranded target each at various concentrations from 0.5 to 20 μM in 50 mM NaCl, 10 mM $MgCl_2$ and 10 mM buffer: (pH 6.4 and 7.0) or T_M values in the tables are reported for oligonucleotide concentrations of 1 μM. Solutions were heated in 1 °C steps, and absorbances were recorded after temperature stabilization using an AVIV 14DS spectrophotometer. Absorbance and temperature readings were plotted using Igor Pro software. T_M values were determined from first-order derivatives as well as graphically from absorbance vs temperature plots. Differences greater than ±1 °C were not observed when using the two procedures. Histeresis experiments were performed by cooling the solutions in 1 °C steps with corresponding absorbance readings. Thermodynamic parameters were obtained using a van't Hoff analysis from plots of $1/T_M$ vs log C for the concentration range of 0.5 to 20 μM.[39,40]

Fluorescence Data. Fluorescence spectra were obtained for single-stranded conjugates or triplex conjugates at concentrations of 1 μM in 50 mM NaCl, 10 mM $MgCl_2$ and 10 mM PIPES, pH 6.4 at ambient temperature. Excitation occurred at 500 nm and emission was observed in the range of 520−570 nm.

Results

The design of the hairpin triplexes relied upon a DNA or RNA sequence that binds to the single-stranded target by both Watson−Crick (W−C) hydrogen bonding as well as by Hoogsteen hydrogen bonding.[4,6] This format requires that the probe sequence bind in an antiparallel manner for the W−C interactions and then make a 180° turn in order to bind in a parallel manner to the Hoogsteen face of the target strand. In previous studies this turn is facilitated by the use of five T residues (Figure 1a) or by the replacement of the T residues by simple linker such as hexa(ethylene glycol).[4−6] We opted to design a simple linker tethering the two different binding sequences that could itself contribute to enhancing complex stability. The design recognized that at the hairpin turn, the triplex structure provides a terminal base triplet that is largely planar in nature and could be envisaged as a target for hydrophobic base stacking or intercalative interactions by an appropriate planar linker (Figure 1b). To take advantage of such targeting we have examined both naphthalene diimide and perylene diimide derivatives as potential planar hydrophobic linkers. These simple aromatic molecules were more readily available than others reported in the literature[12] to be specific for triplex binding. The design of the nucleic acid conjugates employed either a DNA or an RNA target sequence, a DNA/RNA strand for Watson−Crick base pairing, an internally tethered naphthalene or perylene linker followed by a second DNA/RNA strand for Hoogsteen base pairing (Figure 1c).

Synthesis. The linkers were prepared as DMT-protected phosphoramidites such that they could be used in conventional solid-phase DNA synthesis. The synthesis of the various perylene intermediates was complicated by low solubility in most solvents. A significant improvement resulted when at least one of the *t*BDMS- or DMT-protecting groups remained attached to at least one of the linker arms throughout the synthesis. Solubility of the perylene chromophore was no better

(39) Petersheim, M.; Turner, D. *Biochemistry* **1983**, *22*, 256−263.
(40) Marky, L. A.; Breslauer, K. J. *Biopolymers* **1987**, *26*, 1601−1620.

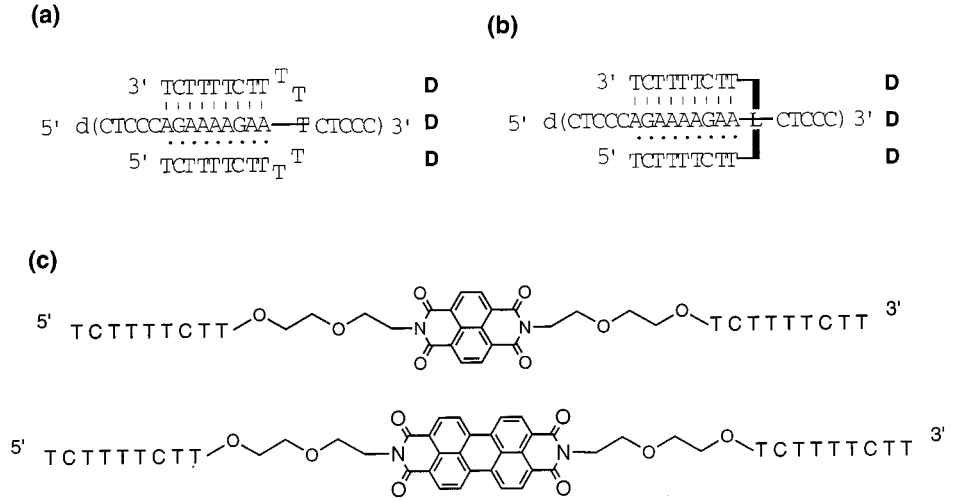
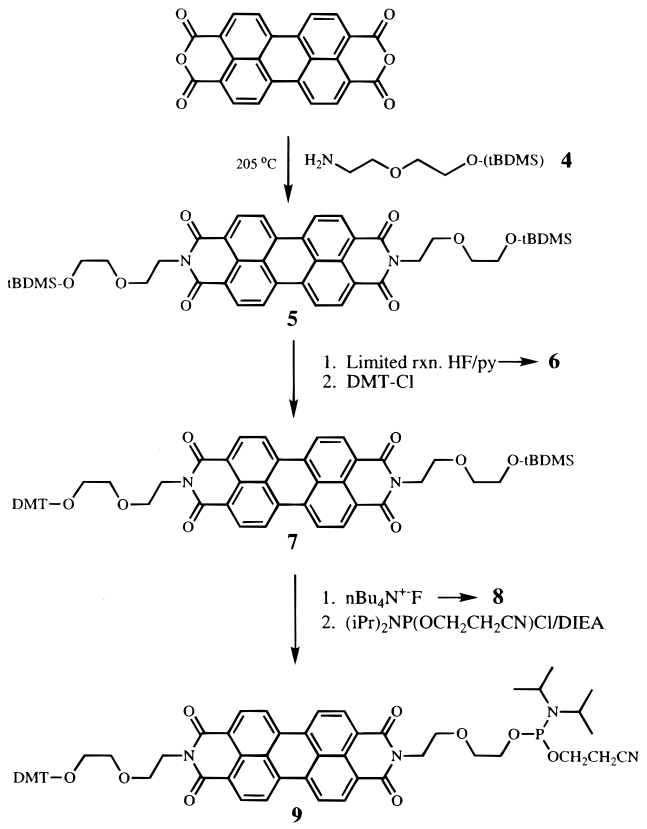


Figure 1. (a) Hairpin triplex with five dT residues tethering the two pyrimidine binding sequences. (b) Complex illustrated in (a) with a non-nucleoside linker tethering to two binding sequences. (c) A DNA−naphthalene diimide conjugate. (d) A DNA−perylene diimide conjugate.

Scheme 1



in aqueous solutions (quite likely worse), but after incorporation into sequences of DNA, the polyanionic nature of the DNA resulted in a significant improvement in aqueous solubility of the conjugates.

A suspension of 3,4,9,10-perylene tetracarboxylicdianhydride (Scheme 1) was treated with 2-(2-*O*-*tert*-butyldimethylsilyl-ethoxy)-ethylamine (**4**) at elevated temperature in the presence of zinc acetate to generate the target diimide **5**. In principle it was only necessary to remove the *t*BDMS-protecting groups and convert this compound to the DMT-protected phosphoramidite. However, upon removal of the *t*BDMS-protecting groups (TBAF) an insoluble solid precipitated. Similar results were obtained in attempting to do a limited deprotection with TBAF. An appropriately soluble product **6** (containing one remaining *t*BDMS-protecting group) could be obtained with a limited HF/pyridine reaction. The mono-*t*BDMS-protected product (**6**) could then be converted to the DMT-protected derivative **7**. The second *t*BDMS-protecting group was then be removed, and the resulting product (**8**) converted to the phosphoramidite derivative (**9**).

The naphthalene linker was prepared in a similar but more straightforward manner. After incorporation of the two *t*BDMS linkers, the *t*BDMS-protecting groups were both removed, and the product was converted successively to the mono-DMT derivative and then to the corresponding phosphoramidite.

Sequences of DNA or 2′-*O*-methyl RNA, or appropriate chimeric sequences were prepared in the conventional manner on CPG solid-phase supports. At the site of the linker, the naphthalene-based phosphoramidite was incorporated in much that same manner as a nucleoside derivative. The perylene derivative continued to exhibit poorer solubility characteristics, and to avoid clogging the lines of the DNA synthesizer, the coupling was performed with the CPG in a small flask. After this coupling reaction, further elongation in the normal manner was also not effective. The first coupling after incorporation of the perylene diimide needed to be performed in a 50:50 dichloromethane:acetonitrile solution. Presumably the solubility of the tethered perylene was problematic in acetonitrile. After the coupling reaction, and the removal of the excess linker, the support became yellow (naphthalene) or burgundy (perylene) in color. After completion of the synthesis, the CPG beads were treated with concentrated methanolic ammonia (naphthalene) or concentrated aqueous ammonia (perylene) overnight. After isolation by PAGE, each complex was analyzed by HPLC with detection at 260 nm and either 383 nm (naphthalene) or 537 nm (perylene). All of the conjugates eluted as single peaks under both conditions of detection.

We were concerned about the stability of the diimide linkages and after isolation subjected the conjugates to conditions of concentrated aqueous ammonia at 50 °C. The perylene conjugates were essentially insensitive to these conditions, but the naphthalene conjugates were readily degraded. After approximately 4 h under these conditions, little intact conjugate remained. Owing to this sensitivity, we performed the deprotection of the naphthalene linkers in methanolic ammonia.

Effects of Naphthalene and Perylene Linkers on Hairpin Triplex Stability. In our initial contribution[36] we reported only on the T_M values for triplexes composed entirely of DNA

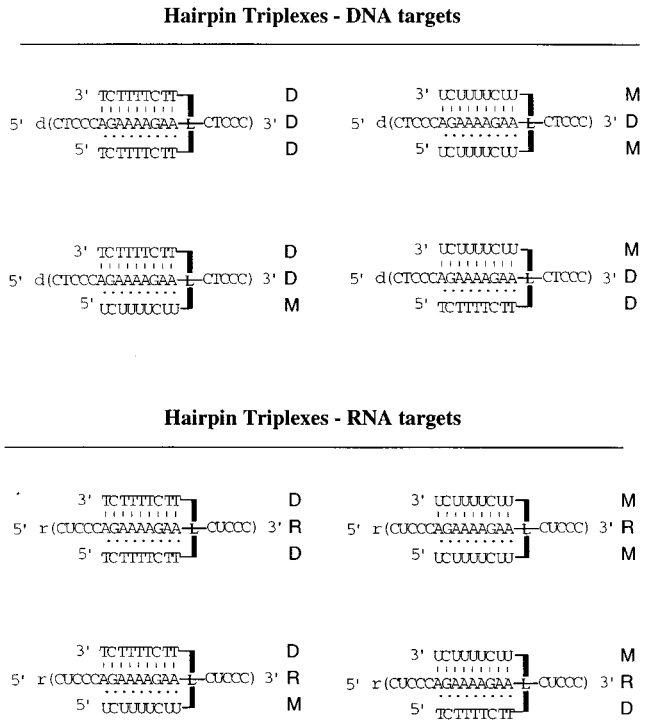


Figure 2. Eight hairpin triplexes formed with a DNA (D) target (upper grouping) or an RNA (R) target (lower grouping) and binding sequences that are both DNA (D), both 2′-*O*-methyl RNA (M) or one strand of each.

sequences and either linker. These represent perhaps the simplest of the possible complexes. Although the hairpin triplexes are composed of only two sequences, there are still three strands of nucleic acid involved in the complex. The nonconjugated sequence can be considered as the "target" strand, and the conjugate binds to the target with two strands, one a Watson−Crick strand (antiparallel) and the second a Hoogsteen strand (parallel). It is then possible to study two groups of complexes, those that have a DNA target strand (D) and those that have an RNA target strand (R) (Figure 2). Within those two groups eight complexes were generated (four with the naphthalene linker and four with the perylene linker), one contains DNA for both the Watson−Crick and Hoogsteen strands, while a second contains RNA (in this case the 2′-*O*-methyl analogue) for both strands. Additional complexes were formed with one DNA strand and one 2′-*O*-methyl RNA strand (M). In total eight conjugated triplexes were prepared for each linker (Figure 2).

The triplexes studied contained a target strand of 19 residues and the polypurine target site occupied the central 9 residues. This target sequence contained two G−C base pairs and seven A−T base pairs. We employed T (or U) in the third strand for A−T base pair targets, and C for G−C base pair targets. In the latter case protonation is necessary to permit bidentate binding to the target G, so we obtained T_M values at pH values of 6.4 and 7.0. In all cases a single thermally induced transition was observed for the conjugated triplexes (Figure 3a), and the cooperativity observed suggested that the transition could be approximated by a two state model. While triplexes formed from a target duplex and a third single strand commonly result in two transitions (triplex → duplex and duplex → random coil) hairpin triplexes formed from a single strand target and a hairpin second/third strand more commonly result in a single transition.[4,6,7] In the present case this observation likely results from the fact that the Watson−Crick and Hoogsteen strands are tethered to one another so denaturation of both strands occurs

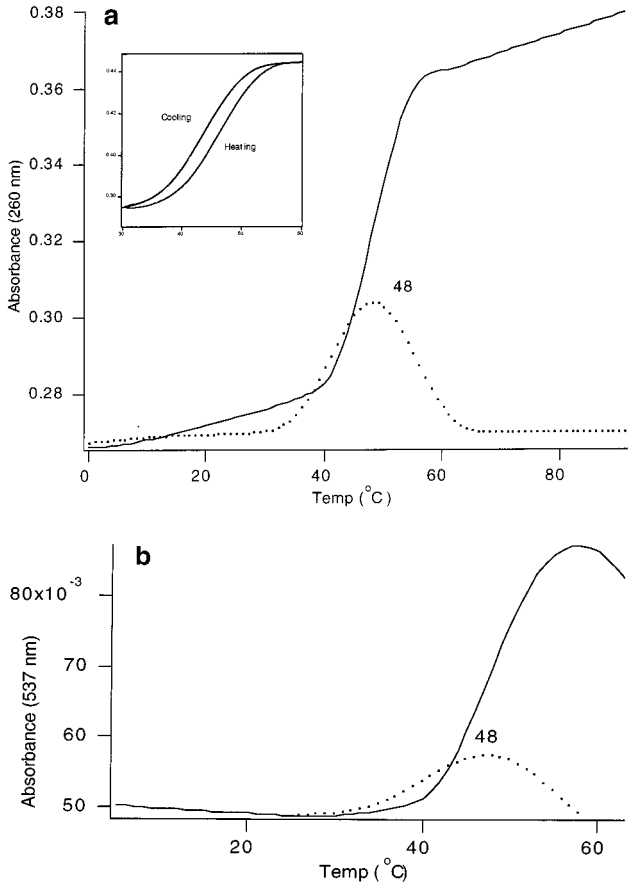


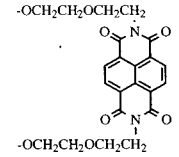
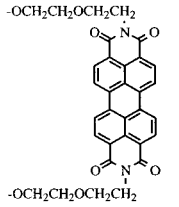
Figure 3. (a) Absorbance vs temperature plot (at 260 nm) for the D−D−D hairpin triplex containing a perylene diimide-based linker. Inset shows hysteresis for the heating and cooling experiments. (b) The same temperature vs absorbance plot as in (a) monitored at 537 nm.

as a single event. The observed transitions were monitored in both "heating" and "cooling" experiments with only a moderate hysteresis effect present (Figure 3a, inset). We additionally monitored the transitions at 383 nm (naphthalene) or 537 nm (perylene) and observed very similar transitions with midpoints essentially identical with those monitored at 260 nm (see Figure 3b). These data further suggested that the denaturation of these conjugate triplexes could be approximated by a two state model. For the conditions of pH 6.4 we employed a van't Hoff analysis[29,30] to obtain free energy parameters for the transitions of sixteen different complexes.

The first group of eight complexes described (Table 1) are those containing a DNA target strand. For comparative purposes we have also prepared a hairpin triplex with a simple hexa-(ethylene glycol) linker. The T_M values for the all DNA triplex formed with this nonplanar simple linker were 24 and 31 °C, at pH values of 7.0 and 6.4, respectively, and the ΔG_{37} determined at pH 6.4 was −7.7 kcal/mol. When the hexa-(ethylene glycol) linker was replaced with naphthalene diimide [and two tri(ethylene glycol) chains] a 6 kcal/mol change in ΔG_{37} was observed. With the perylene diimide present, a further 1.3 kcal/mol of stabilization results, some 7.3 kcal/mol more stable than the complex containing the simple glycol linker. As the nature of the backbones (DNA vs RNA) of the Watson−Crick and Hoogsteen strands were varied, notable changes in stability were observed. The most dramatic difference occurred when both strands were 2′-*O*-methyl RNA. This complex, with a T_M increase of 28 °C, exhibited 21 kcal/mol of stabilization, some 13.3 kcal/mol more stable that the complex containing the simple glycol linker. The mixed DNA/RNA complexes

Table 1. Stability of Triplexes Containing Naphthalene- and Perylene-Based Linkers with a DNA Target Strand[a]

Sample Triplex/19-mers (DNA targets):

Linker = L		Complex[b]			
	W.-C Strand →	D	D	M	M
	Target Strand →	D	D	D	D
	Hoogsteen Str. →	D	M	D	M
-O(CH$_2$CH$_2$O)$_5$CH$_2$CH$_2$O-	pH 7.0 (°C)	24			
	pH 6.4 (°C)	31			
	ΔG$_{37}$ (kcal/mole)	-7.7			
	ΔH (kcal/mole	-70			
	ΔS (cal/M°K)	-203			
	pH 7.0 (°C)	36	48	37	46
	pH 6.4 (°C)	45	50	39	51
	ΔG$_{37}$ (kcal/mole)	-13.7	-14.3	-9.9	-15.4
	ΔH (kcal/mole	-162	-115	-118	-172
	ΔS (cal/M°K)	-480	-326	-351	-505
	pH 7.0 (°C)	44	49	37	56
	pH 6.4 (°C)	48	55	43	59
	ΔG$_{37}$ (kcal/mole)	-15.0	-17.7	-10.3	-21.0
	ΔH (kcal/mole	-153	-161	-94	-179
	ΔS (cal/M°K)	-445	-461	-268	-509

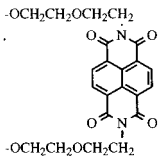
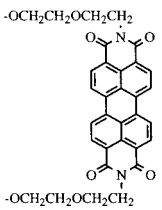
[a] T_M values were obtained for duplexes or complexes containing a 1:1 mixture of oligonucleotide−linker−oligonucleotide and single-stranded target in PIPES at the noted pH at oligonucleotide concentrations of 1 μM. Solutions were heated in 1 °C steps, and absorbances were recorded after temperature stabilization using an AVIV 14DS spectrophotometer. Absorbance and temperature readings were plotted using Igor Pro software. T_M values were determined from first-order derivatives as well as graphically from absorbance vs temperature plots. T_M values are the average of at least two determinations reproducible to ±1 °C. ΔG_{37} values were calculated for the data at pH 6.4 from $1/T_m$ vs log C plots. [b] D = oligodeoxynucleotide, M = 2′-*o*-methyl-oligoribonucleotide.

exhibited 17.7 and 10.3 kcal/mol of complex stabilization, the more stable complex occurring when both strands of the target duplex were DNA and the Hoogsteen strand was RNA.

The second group of eight complexes (Table 2) are those containing an RNA target strand. In these cases the triplex was much less stable. For the complex containing a hexa(ethylene glycol) linker, no transition was observed at pH 7.0, and at pH 6.4 a very early transition was observed with an estimated T_M of about 10 °C. However, this transition was not sufficiently distinct to permit thermodynamic characterization. The stabilizing effect of the planar linkers resulted in much more stable complexes that could be effectively characterized. In the best case (the all RNA triplex) the T_M value was increased by roughly 36 °C with 10.8 kcal/mol of stabilization. By comparison, when two DNA strands were used with the RNA target, a more moderate increase in T_M of about 12 °C was observed (ΔG_{37} = −7.8 kcal/mol). With both linkers, those complexes containing at least one RNA strand were more effective in stabilizing the triplex with the RNA target than the conjugate containing two DNA strands.

Table 2. Stability of Triplexes Containing Naphthalene- and Perylene-Based Linkers with an RNA Target Strand[a]

Sample Triplex/19-mers (RNA targets):

Linker = L		Complex[b]			
	W.-C Strand →	D	D	M	M
	Target Strand →	R	R	R	R
	Hoogsteen Str. →	D	M	D	M
-O(CH$_2$CH$_2$O)$_5$CH$_2$CH$_2$O-	pH 7.0 (°C)	nd[c]			
	pH 6.4 (°C)	~10[d]			
	ΔG$_{37}$ (kcal/mole)	–			
	ΔH (kcal/mole	–			
	ΔS (cal/M°K)	–			
	pH 7.0 (°C)	21	39	37	41
	pH 6.4 (°C)	22	40	40	42
	ΔG$_{37}$ (kcal/mole)	-7.8	-9.6	-9.7	-10.0
	ΔH (kcal/mole	-34	-54	-35	-55
	ΔS (cal/M°K)	-87	-143	-82	-144
	pH 7.0 (°C)	28	38	38	45
	pH 6.4 (°C)	29	39	43	46
	ΔG$_{37}$ (kcal/mole)	-7.9	-9.9	-10.3	-10.8
	ΔH (kcal/mole	-47	-73	-47	-63
	ΔS (cal/M°K)	-126	-204	-117	-170

[a] See footnote *a* of Table 1. [b] See footnote *b* of Table 2. [c] No transition detected. [d] Transition begins <0 °C, T_M is approximated.

In general the larger perylene linker was more effective than the naphthalene in enhancing the stabilization of the three stranded complexes. The largest difference between the two planar linkers was 5.6 kcal/mol observed for the complex formed from a DNA target and a conjugate containing two RNA strands (Table 1). By comparison, all of the complexes containing an RNA target had very similar ΔG values regardless of the linker employed.

Fluorescence Properties. Perylene derivatives have well-documented fluorescence properties that were useful in characterizing some of the conjugated triplex properties. Neither the perylene tetraacetic dianhydride, nor any of the linker-only derivatives prepared, could be used to obtain usable fluorescence spectra in aqueous solution. Either the solubility of these derivatives was so poor that no significant amounts of material were solubilized, or material in solution underwent self-aggregation and resulted in self-quenching of any fluorescence emission properties. However, upon conjugation to the charged nucleic acids, the DNA/RNA−perylene linkers could be dissolved in water and fluorescence spectra were obtained. With an excitation wavelength of 500 nm, the perylene conjugates exhibited an emission maximum at 546 nm. The quantum yields for various conjugates exhibited significant variations (Figure 4a). The conjugate containing two RNA strands (as the 2′-*O*-methyl derivatives) resulted in the highest quantum yield, while that containing two DNA strands the lowest (reduced some 6-fold). The conjugate containing one RNA and one DNA strand was intermediate in effect.

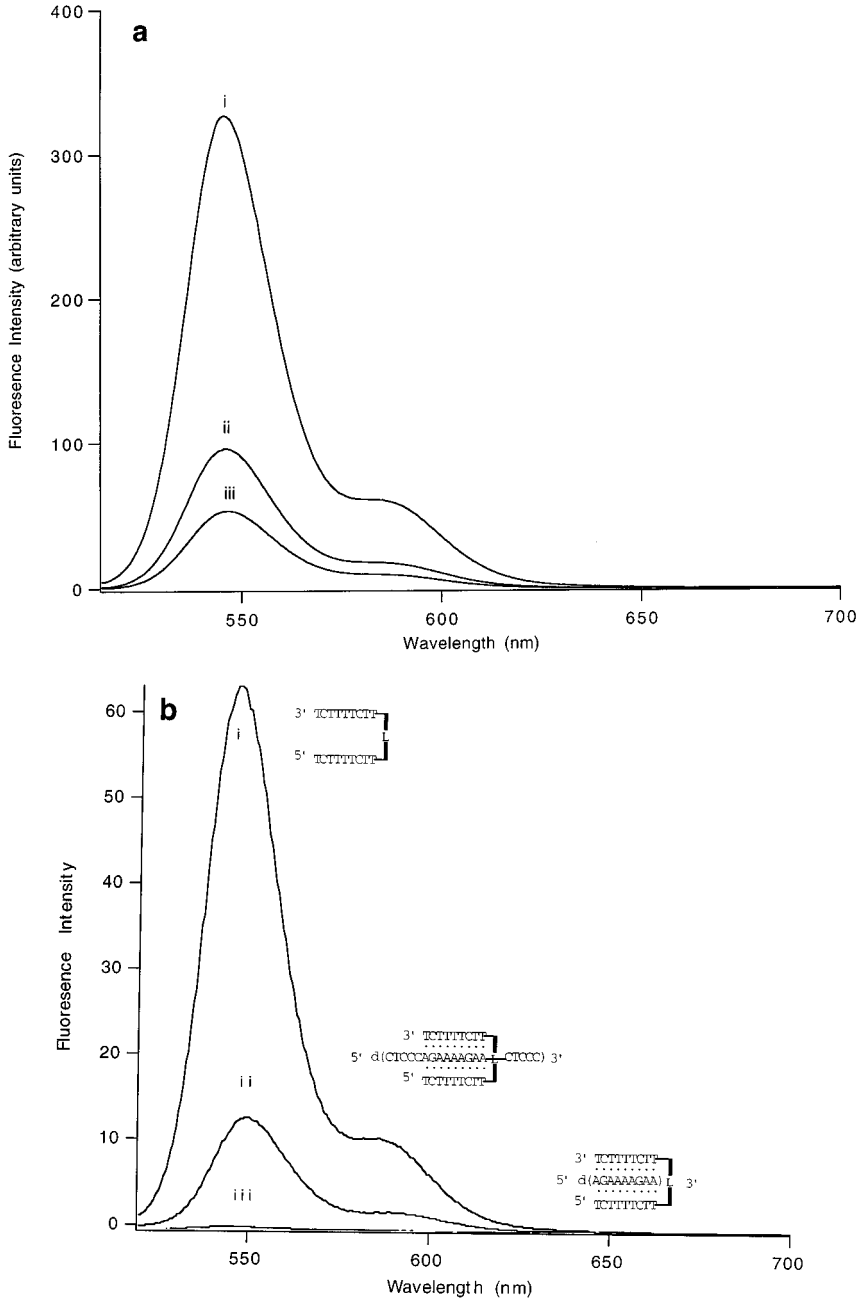


Figure 4. Fluorescence spectra of various perylene conjugates obtained at concentrations of 1 μM and pH 6.4. (a) Fluorescence emission spectra for (i) the 5′-RNA−perylene−RNA conjugate, (ii) the 5′-RNA−perylene−DNA conjugate, (iii) the 5′-DNA−perylene−DNA conjugate, all obtained at concentrations of 1 μM. (b) Fluorescence emission spectra for (i) the 5′-DNA−perylene−DNA conjugate, (ii) the 5′-DNA−perylene−DNA conjugate with the DNA 19-nucleotide target, and (iii) the 5′-DNA−perylene−DNA conjugate with the purine-only 9-nucleotide target, all obtained at concentrations of 1 μM.

Upon complexation to the target strand the emission of the perylene linker in the all DNA complex was quenched to some 14% of its initial value (Figure 4b). Some variation in the quenching effects were observed for the other seven complexes (Figure 5). During the course of these studies we also prepared a smaller triplex complex, one composed of the shortest possible target, a 9-mer. The T_M values obtained for the all DNA complex with this 9-mer were similar to those obtained with the 19-mer target, but the fluorescence effects were quite different. With this complex almost complete quenching of the emission signal was observed. Both of these experiments, with either the 19-mer or the 9-mer target, are consistent with energy transfer between the perylene and stacked base residues of the triplexes. Quenching of the perylene excited state by aromatic pi systems has been observed previously.[41]

Discussion

The goal of this study was to target the base triplet at the terminus of the three-stranded complex formed from a hairpin triplex as a site to enhance hydrophobic base-stacking interactions and thereby provide additional overall stabilization to the complex. The design of both linkers employed was based upon aromatic diimides, one a derivative of naphthalene and the second originating from perylene. The synthetic scheme for the former was relatively straightforward, while that for the latter

(41) Icli, S.; Icil, H.; Gurol, I. *Turk. J. Chem.* **1997**, *21*, 363−368.

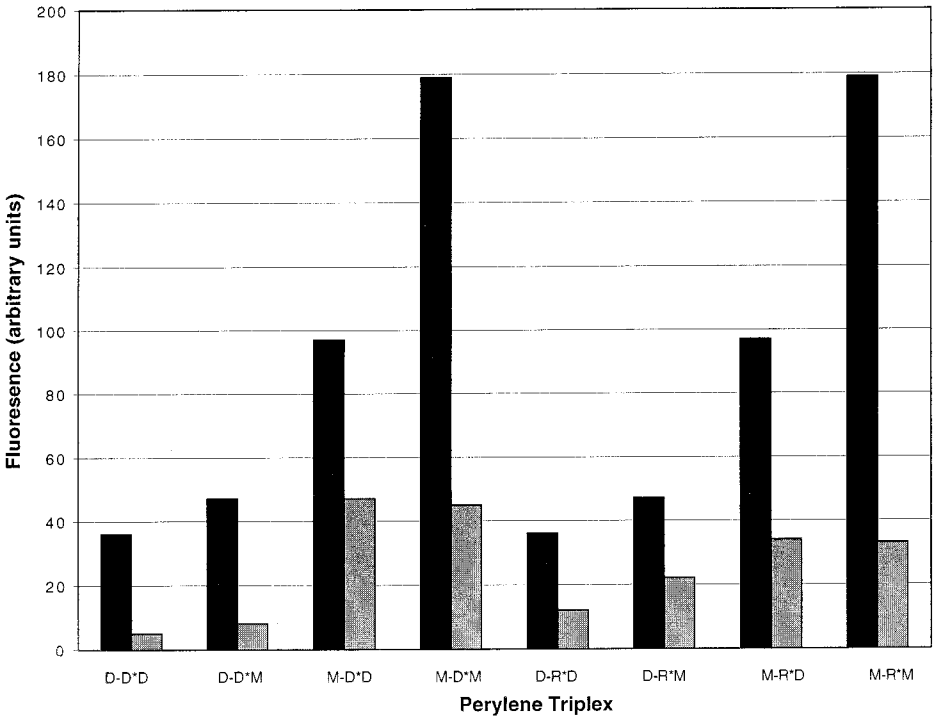


Figure 5. Bar graph illustration of the relative fluorescence quantum yields for the simple uncomplexed DNA/RNA conjugates containing a perylene-based linker (black) in comparison with the corresponding triplexes (gray) obtained by titrating the perylene-based conjugates with one equivalent of the DNA or RNA target strand. For example, D−R*D represents the Watson−Crick*Hoogsteen complex with an RNA (R) target and two DNA (D) strands from the perylene conjugate.

was complicated by the poor solubility characteristics of the perylenes in general. However, appropriate selection of protection groups permitted us to convert the perylene diimide into the desired phosphoramidite, and appropriate choice of coupling procedures permitted the preparation of the complexes in sufficient quantities. Isolation by HPLC methods of the perylene-based conjugates was complicated by the hydrophobic nature of the linkers, but PAGE isolation eliminated these complications and was an effective alternative.

The perylene-based linker is more likely to be effective in triplex stabilization since modeling studies suggest that it can potentially provide more effective stacking interactions with all three base residues of the terminal base triplet (Figure 6). This expectation was observed for those complexes involving a DNA target. Less differentiation was present with complexes containing an RNA target suggesting the possibility that in these latter complexes the perylene linker was not positioned in such a manner to maximize stacking interactions. With the described 19-mer the linker must "cross" the target strand at some point in order for both Watson−Crick and Hoogsteen interactions to take place. With the described complexes there is the possibility that the first residue extending beyond the triplex complex may be stacked onto either the naphthalene- or perylene-based linker at the point in which the linker crosses the target strand in such a manner that the linker would effectively be intercalated between the last base triplet and the first single-stranded base of the target. In the complex illustrated here the first single-stranded base beyond the last triplet is a cytosine residue, which typically has the poorest of base-stacking interactions of the four common bases. By comparison, adenine has demonstrated the strongest base-stacking interactions. As we reported in our initial contribution,[36] when the target sequence was changed to effect a substitution of dA for dC at this site, an additional 3 °C increase in T_M was observed. This result suggests a complex with the naphthalene or perylene derivative sandwiched between the terminal base triplet and the next single-stranded base.

Base-stacking interactions between the planar linkers and the nucleic acid triplexes are further indicated in that (i) absorbance vs temperature plots obtained at 383 or 537 nm for duplexes containing the naphthalene- or perylene-based linkers, respectively, also exhibited identical cooperative increases in hyperchromicity with a midpoint in the transitions that corresponded to those obtained at 260 nm (see Figure 3a and 3b). (ii) Additionally, absorbance vs temperature plots obtained in solutions containing 10−30% ethanol, resulted in T_M reductions from 3 to 7 °C as we described in our initial contribution.[36] While these experiments suggest that stacking interactions between the linkers and the nucleic acids bases are present, ethanol can also destabilize nucleic acid complexes in the absence of any aromatic linkers by interfering with base-stacking interactions.

T_M and ΔG values varied for the complexes, and were dependent on the nature of the DNA or RNA composition of the complexes. For the DNA target, with both conjugate strands containing 2′-*O*-methyl RNA (i.e., M−D*M = Watson−Crick*Hoogsteen strands), the T_M was increased by as much as 12 °C for the naphthalene linker and as much as 16 °C for the perylene linker (relative to conjugates in which one or both strands are DNA). ΔG values for the M−D*M complex containing the naphthalene- and perylene-based linkers were −15.4 and −21.0 kcal·mol^{-1}, respectively. By comparison, ΔG values for the M−D*D complexes were −9.9 (naphthalene linker) and −10.3 (perylene linker) kcal·mol^{-1}. The T_M and ΔG data therefore suggest that the complexes in which both probes are 2′-*O*-methyl RNA are the most stable. One mixed probe strand containing the naphthalene linker (D−D*M) had a T_M similar to that of the M−D*M complex with the same linker. This trend has been observed previously where it was reported[42] that R−D*R and D−D*R followed by D−D*D were the most stable complexes formed. In other studies, and in the presence of 2′-*O*-methyl RNA, the following relative stabilities: M−D*M

(42) Roberts, R. W.; Crothers, D. M. *Science* **1992**, *258*, 1463−1466.

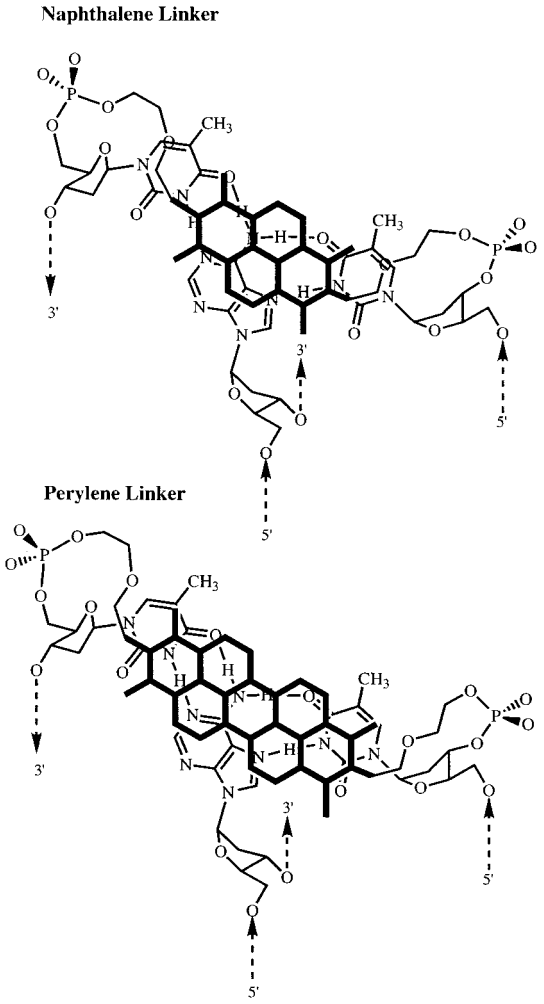


Figure 6. Illustration of the approximate relationships of the naphthalene linker (upper) and perylene linker (lower) linkers to the terminal base triplet of the hairpin triplex.

> D−D*M > D−D*D > M−D*D were reported.[43] Other studies[44] have suggested much different relative orders, and these differences may be due to variation in the sequences employed or in the experimental conditions. Previous studies determined the ΔG values for similar complexes containing the hexa(ethylene glycol) linker.[4] For the hexa(ethylene glycol) sequence illustrated in Table 1 (with an all DNA conjugate) the ΔG_{37} was determined to be −7.6 kcal·mol^{-1}. This value is comparable to that previously determined for a similar complex and is consistent with our finding that complexes containing this simple glycol linker are less stable than those containing the naphthalene- and perylene-based linkers.

Tables 1 and 2 also indicate that the T_M values for complexes containing a perylene-based linker are 4−8 °C higher than the corresponding complex with a naphthalene-based linker (at pH 6.4). The corresponding ΔG values correlate well with these data in that for each type of complex the ΔG values were slightly more negative for the complexes containing the perylene-based linkers. Complex formation appears to be enthalpy-driven in all cases, but the differences in stability between the naphthalene and perylene complexes for the DNA target arise in part from a slightly less unfavorable entropy term for the perylene complexes. The data indicate that the perylene linker is better able to stabilize complexes containing the DNA target than the naphthalene linker. We also note that the T_M values at pH values of 6.4 and 7.0 do not differ much for the RNA target series. Since we were unable to obtain effective transitions for those complexes containing the hexa(ethylene glycol) linker, the presence of the planar linker appears to be a dominating force in the formation of this series of complexes.

For complexes with a RNA target the results are less dramatic. The T_M values obtained for the D−R*D complex were 22 and 28 °C for the naphthalene- and perylene-based linkers respectively (the lowest T_M values observed in this study). ΔG_{37} values for the DR*D complexes were −7.8 and −7.9 kcal/mol (the lowest ΔG_{37} values observed in this study). A general rule for DNA/RNA mixed triplexes seems to be that if the duplex contains the R purine strand, only the R third strand will bind. T_M values for the M−R*M complexes were 17−20 °C higher than those for the D−R*D complexes, consistent with this observation. Again, the ΔG_{37} values correlated well with the T_M values (2.2−2.9 kcal·mol^{-1} more negative than those for the D−R*D complexes), which indicates that the all-RNA probe is significantly more stabilizing when the target is RNA than the all-DNA probe. The mixed probes demonstrated intermediate values of T_M and ΔG. The order of stability previously reported from most stable to least is R−R*R > R−R*D > D−R*R, D−R*D; and if 2′-*O*-methyl made up one or two of the pyrimidine strands, the order was M−R*M > M−R*D > D−R*M > D−R*D.[35] This pattern seems similar to the pattern observed in these experiments; however, in that previous work, circular oligonucleotides were used in which the loop portions were penta-nucleotides.

In comparing the naphthalene-based and perylene-based linker complexes containing the RNA probe, again in each case the T_M values for the complexes containing a perylene-based linker are only slightly higher than those for the corresponding complex with a naphthalene-based linker. The corresponding ΔG_{37} values are also quite similar regardless of the linker. These results suggest that with the RNA target the perylene linker may be less optimally positioned, or that the terminal base triplet is less coplanar, such that effective stacking interactions with the larger perylene-based conjugate are not maximized.

Nearly all effective DNA intercalators are not only planar in geometry but also contain a positive charge. The former property permits the agent to "slide" between base pairs, while the latter may be important for charge−charge interactions with neighboring phosphates and necessary to lock the intercalator into position. Other types of planar molecules lacking a positive charge, most notably psoralen and the diol-expoxide derivatives of benzopyrene, have not been observed in fully intercalated states until they are covalently tethered to the duplex. After alkylation, the intercalated mode of binding appears to predominate.[45,46] Such ligands can be considered to be transient intercalators in the absence of covalent bond formation. The planar linkers described in this study also lack any positive charge; however, the presence of significant binding interactions with the tethered Watson−Crick and Hoogsten pyrimidine strands will certainly position the planar intercalating linkers in position to be effectively stacked on the terminal base triplet, and intercalated between the triplet and the next base of the single-stranded target. The energetics of binding by selected positively charged intercalators has been determined, and for

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two quite different agents, ethidium bromide and daunomycin, the free energies for intecalative binding are essentially identical at −9 kcal/mol.[47] With the present complexes, the difference between the hexa(ethylene glycol) linker and the planar linkers can be roughly compared. And although it is unlikely that the energetic effects are strictly additive, this comparison (for the D−D*D complex) suggests that the intercalating linkers provide roughly 6 kcal/mol (naphthalene) and 7.3 kcal/mol of stabilization energy. These values are probably not unreasonably different from the ethidium bromide and daunomycin values, given that those agents when intercalated experience $\pi-\pi$ interactions to both planar surfaces, while with the present complexes the $\pi-\pi$ interactions largely occur to one of the aromatic faces.

The large increase in complex stability between the control complex containing the hexa(ethylene glycol) linker and those containing the naphthalene and perylene linkers appears to be largely due to the stacking interactions afforded by the latter planar derivatives. However, other aspects may also contribute to this enhanced stability. The planar linkers have fewer rotatable bonds, relative to hexa(ethylene glycol) so that fewer unfavorable conformations may be present for the planar linkers. Additionally, while the planar linkers are designed to interact favorably with the target strand, the simple hexa(ethylene glycol) linkers offers no favorable interactions, and may in fact be presented with unfavorable steric challenges as it must cross the target strand in its role of tethering the Watson−Crick and Hoogsteen binding oligonucleotides. Other parameters clearly affect complex stability, the most obvious being the mixture of DNA/RNA within the complexes. Regardless of the target strand (DNA or RNA) the most stable complexes in each series resulted when both the Watson−Crick and Hoogsteen strands were RNA or 2′-*O*-methyl RNA (M−D*M and M−R*M complexes). By comparison, the least stable complexes in each series were generally those in which a Watson−Crick heteroduplex was formed with a third strand of DNA (M−D*D and D−R*D complexes). Although the structures of these various triple helices have eluded formal explanation by X-ray analyses, it is clear that base−base interactions alone cannot explain these differences.

The quantum yields from the fluorescence experiments for the perylene-based conjugates are dependent upon the nucleic acid composition. The all-RNA conjugate is likely the most polar of the conjugates, owing to the presence of the 2′-OH on each sugar residue and the absence of the methyl group on the uracils. In the RNA−DNA conjugate, the uracils of the DNA strand are methylated as thymines. The presence of the methyl groups and the absence of the 2-OH at each site results in a more hydrophobic sequence, and one that is likely more capable of stacking or otherwise interacting with the hydrophobic perylene linker. Any kind of base-stacking event will likely result in energy transfer, and as observed, the conjugate with two DNA strands is much less fluorescent than that with two RNA strands.

The quenching observed upon complex formation is most dramatic for the minimum 9-nucleotide target, but still impressive for the 19-mer target (Figures 4b and 5). Although quenching was observed in all cases, significant variations were present. Within the RNA target series, the smallest quenching effects were observed for the M−R*D complex (46% of conjugate), and this is also the least stable triplex (see Table 2). The most dramatic quenching effects were observed for the all-M−R*M triplex (18% of conjugate); this complex is also the most stable of the four triplexes formed from RNA target strands. The remaining two triplexes have intermediate quenching effects and exhibit intermediate ΔG values (compare Figure 1 with Figure 5). The results with the DNA target are less conclusive. The smallest quenching effects were observed for the M−D*D triplex (48% of conjugate), and this complex also exhibited the lowest ΔG value for the DNA target series (Table 1). However, the most significant fluorescence quenching was observed for the D−D*D and D−D*M triplexes (14 and 17% of conjugate, respectively), while the most thermodynamically stable triplex is the M−D*M complex (with 25% fluorescence relative to the conjugate). This inconsistency may simply reflect some preference for stacking on the DNA−DNA Watson−Crick duplex that is only present in these two complexes. With the DNA−DNA duplex present, the fluorophore stacks onto a dA-dT base pair. Since the quantum yield for the single-stranded conjugates is most dramatically reduced for the DNA−linker−DNA sequence, the presence of the dT residues likely stack more effectively with the linker and result in more dramatic quenching whether in simply the single-stranded conjugate or the corresponding three-stranded complex. Such an effect would skew the relationship between triplex stability, which is the result of both the nature of the three nucleic acid strands and of the stacked ligand, while the fluorescence quenching data only reflects the nature of the interaction of the perylene with, presumably the terminal base triplet.

While variations in the fluorescence quenching effects are likely to reflect some slight differences in the nature of the hydrophobic stacking interactions between the fluorophore and the planar aromatic bases, the most likely structure consistent with the observed data is that in which the planar chromophore stacks on top of the terminal base triplet of the three-stranded complex (see Figure 6). That the complex with the shorter 9-mer target is largely non-fluorescent is likely a result of dimerization about the perylene linkers occurring between two triplexes. The additional perylene−perylene stacking as the result of dimerization likely contributes to additional self-quenching effects.

Conclusions

The use of planar naphthalene and perylene diimide linkers to bridge the Watson−Crick and Hoogsteen hydrogen bonding strands in a hairpin triplex imparts significant additional stability to the three-stranded complex. These types of conjugates can be used effectively to target single-stranded nucleic acids and could be effective modulators of genetic expression by complexing to single-stranded mRNA or single-stranded portions of DNA actively involved in transcriptional processes.

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