Use of a Stilbenedicarboxamide Bridge in Stabilizing, Monitoring, and Photochemically Altering Folded Conformations of Oligonucleotides

Robert L. Letsinger* and Taifeng Wu

Contribution from the Department of Chemistry, Northwestern University, Evanston, Illinois 60208

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Abstract: A stilbenedicarboxamide bridge (-Σ-) is shown to serve as a unique and unusually effective cap for stabilizing and characterizing short oligonucleotide double and triple stranded structures. As representative of the stability of a family of oligomers studied, T_m values for the thermal transitions in 0.1 M NaCl of the hairpin forms of dTTT-Σ-dAAA and dGCG-Σ-dCGC are ~42 °C and >80 °C, respectively, and T_m for unfolding of the triple-stranded, double-hairpin conformation of d(TTTTTT-Σ-)$_2$dAAAAAA is 69 °C. The intercalating dye, ethidium, binds efficiently to the mini-hairpin structures formed by these conjugates, even to dTT-Σ-dAA, which has a single four-nucleotide pocket; and the DNA groove binding agent, Hoechst 33258, interacts with dTTT-Σ-dAAA as well as with longer conjugates. The distinctive monomer and excimer fluorescence bands for the stilbenedicarboxamide moiety provide useful information on the structures of these small organized domains, and photoinduced isomerization permits postsynthetic alteration of the geometry of the bridge and therefore alteration of the hybridization properties of the conjugate. The sensitivity to light depends on the nucleotides abutting the linker. When stability to light is desired, it can be achieved by incorporating a dG-dC pair in this position.

Introduction

Intramolecular hairpin conformations constitute important motifs in the folding of RNA and DNA molecules into higher order structures. A number of studies have established that these structural units are associated with diverse biological functions of nucleic acids, such as catalysis,[1] binding to specific regulatory proteins,[2,3] and initiation of assembly of large functional molecular complexes.[4,5] A distinguishing feature of oligonucleotide chains that fold into hairpin conformations is their high thermal stability.[6,7] Several groups have recently shown that non-nucleotide bridges based on oligo(ethylene glycol),[8−10] terephthalamide,[11] and disulfide links[12,13] can be used as caps in place of oligonucleotide segments to stabilize short helical double- or triple-stranded oligonucleotide conformations. The oligonucleotide conjugates containing such bridges have potential as surrogates for polynucleotides in the design of decoys for DNA- or RNA-binding proteins,[14] oligonucleotide-based catalysts,[15,16] and novel self-assembling systems.

We report here that an extended stilbenedicarboxamide bridge (I) is an unusually effective structural element for stabilizing short double- and triple-stranded oligonucleotide domains.[17] This unit was selected for study since it possesses a long, relatively rigid core, which should reduce the entropy of the overall bridge, and it contains a fluorescent, photoresponsive chromophore, which serves both as a reporter group and as a vehicle for triggering changes in geometry. Three-carbon linkers were used to connect the termini of the oligonucleotides to nitrogen atoms of stilbenedicarboxamide so that the overall length would correspond to the distance across a DNA double helix.

Results

Oligomers. The oligonucleotide conjugates prepared for this study along with some pertinent physical properties are listed in Table 1. These compounds were synthesized *via* conventional phosphoramidite chemistry using compound IV as a reagent to introduce the stilbenedicarboxamide linker (see Chart 1). This synthon was obtained by conversion of *trans*-stilbene-4,4′-dicarboxylic acid through the di-acid chloride and diol II to the mono(4,4′-dimethoxytriphenylmethyl) ether derivative, III, which was phosphitilated with $ClP(OCH_2CH_2CN)N(i\text{-}C_3H_7)_2$.

The chromatographic and spectral data for this family of compounds correlate well with expectations based on the specified nucleotide/stilbene ratios and sizes of the oligomers. The hydrophobic stilbenedicarboxamide linker retards migration

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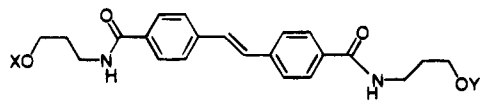
Table 1. Properties of Oligonucleotide–*trans*-Stilbenedicarboxamide Conjugates

compd	RP HPLC[a] (min)	UV spectra[b] A_{260}/A_{334}	T_m[c] (°C)
dT-Σ-dA (**1**)	24.6	0.58	
dTT-Σ-dAA (**2**)	22.5	0.85	
dTTT-Σ-dAAA (**3**)	19.8	1.29	~42
dTTTT-Σ-dAAAA (**4**)	19.0	1.58	49
dTTTTTT-Σ-dAAAAAA (**5**)	18.7	2.08	59[d,e]
dT_{12}-Σ-A_{12} (**6**)	20.0	3.70	64[d]
(dTTTTTT-Σ-)$_2$dAAAAAA (**7**)	21.6	1.65	69
dG-Σ-dC (**8**)	24.6	0.53	
dGC-Σ-dGC (**9**)	21.6	1.08	
dGCG-Σ-dCGC (**10**)	20.6	1.38	>80[f]
dTTGC-Σ-dGCAA (**11**)	20.8	1.68	>70
dTTCT-Σ-dAGAA (**12**)	20.8	1.55	~57
dTTCTTT-Σ-dAAAGAA (**13**)	20.3	2.10	66
dTTTTTT-Σ-dTTTTTT (**14**)	19.0	2.40	
dCCCCCC-S -dCCCCCC (**15**)	18.7	1.67	

[a] Elution time for HPLC (see the Experimental Section). [b] Relative peak intensities for UV spectra at 23 °C; H_2O, 0.1 M NaCl, 10 mM Tris-HCl, pH 7. [c] T_m is the temperature at the midpoint of the dissociation curve, determined for 1.0 A_{260} unit/mL of oligomer in the same solvent as in *b*. [d] T_m values were determined at an oligomer concentration of 0.2 as well as 1.0 A_{260} units/mL; the same values were obtained at both concentrations. [e] For comparison, T_m = 49 °C for a photoproduct from **5**, probably the *cis* isomer. [f] When the solvent was 50% ethanol/water (no salt), the T_m value was 66 °C, for oligomer concentrations of 0.2 A_{260} units/mL and 1.0 A_{260} units/mL.

Chart 1



I. stilbenedicarboxamide linker. X = Y = - OP (O)(O⁻) -

II. diol intermediate. X = Y = - H

III. monodimethoxytrityl derivative. X = DMT - ; Y = - H

IV. phosphoramidite reagent. X = DMT -; Y = - P(N·IPr$_2$)(OCH$_2$CH$_2$CN)

on HPLC, an effect that is most pronounced for the shorter oligomers. The UV absorption spectra exhibit two major bands centered at about 334 and 260 nm; radiation at 334 nm is exclusively absorbed by the stilbene chromophore whereas that at 260 nm is largely absorbed by the nucleotide bases. The ratio of the absorbance values at these wavelengths serves as an indicator of the the nucleotide/stilbene ratio.

Thermal dissociation profiles for a family of dTn-Σ-dAn oligomers are presented in Figure 1. A self-consistent pattern of curves showing increase in thermal stability and hyperchromicity with increasing chain length was obtained. A striking feature is the high temperatures for these transitions. The T_m value for dTTTTTT-Σ-dAAAAAA in aqueous 0.1 M NaCl is 59 °C. Under the same conditions, T_m for dissociation of a complex formed from dTTTTTT + dAAAAAA is <0 °C. The stabilizing effect of the stilbenedicarboxamide bridge is significantly greater than that of a terephthalamide bridge (T_m 42 °C for dTTTTTT-X-dAAAAAA[11] or a tris(ethylene glycol) bridge (T_m 35 °C for dTTTTTT-E_3-dAAAAAA; see the Experimental Section). The hypochromic conformations of dTTTT-Σ-dAAAA and dTTT-Σ-dAAA are also unusually stable, and even dTT-Σ-dAA exhibits an increase in absorbance indicative of base stacking. The hyperchromicity associated with "melting" of the compounds in this family increases with increasing chain length (h = ~22%, 24%, 34%, and 41%, for oligomers with n = 3, 4, 6, and 12, respectively), in accord with the assumption that the hyperchromicity associated with a given internal dT/dA pair (one bounded by nucleotides on both

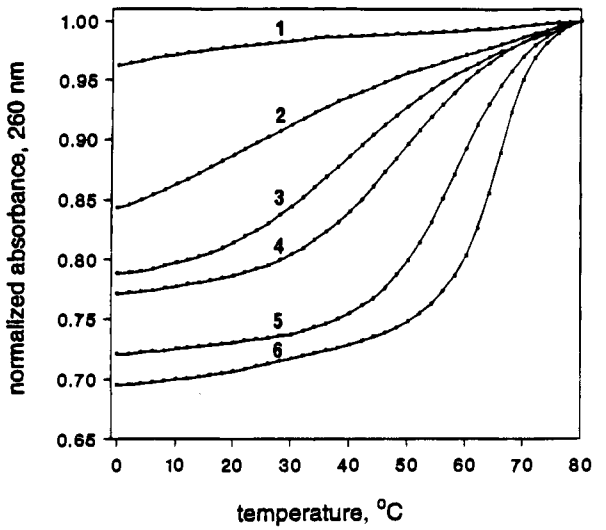


Figure 1. Thermal dissociation profiles in standard buffer (H_2O, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.0) (top to bottom): 1, dT-Σ-dA; 2, dTT-Σ-dAA; 3, dTTT-Σ-dAAA; 4, dTTTT-Σ-dAAAA; 5, dTTTTTT-Σ-dAAAAAA; and 6, dT_{12}-Σ-dA_{12}.

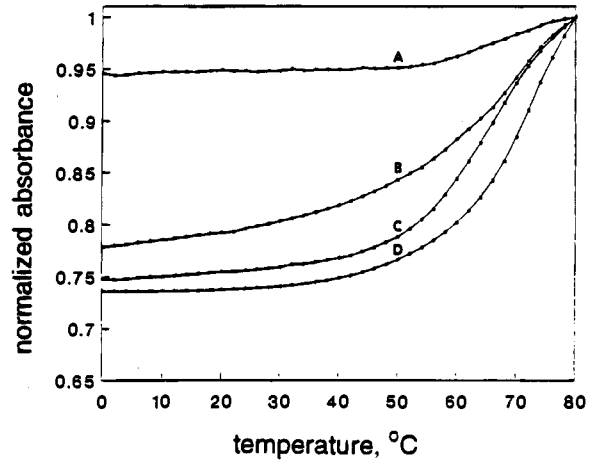


Figure 2. Thermal dissociation profiles in water, 1.0 M NaCl, 10 mM Tris-HCl (pH 7.0) (top to bottom): A, dTTTTTT-Σ-dAAAAAA (**5**), and B, d(TTTTTT-Σ-)$_2$dAAAAAA (**7**), monitored at 280 nm; and C (**5**) and D (**7**) monitored at 260 nm.

sides) is similar to that for a pair in poly(dT)/poly(dA) and is greater than that for the stilbene residue or a terminal dT/dA pair.

Compound **7**, d(TTTTTT-Σ-)$_2$-dAAAAAA, was prepared to see if the stilbenedicarboxamide linker, like the terephthalamide linker[11] would stabilize folded triple-stranded structures. The melting curves in Figure 2 show that **7** in fact forms a complex that is considerably more stable (T_m 69 °C) than the triple-stranded conformation of the corresponding oligomer containing terephthalamide linkers (T_m 58 °C). In addition, the large increase in absorbance in the curve for **7** monitored at 280 nm is indicative of dissociation of a triple stranded dT/dA complex. For comparison, the absorbance change at 280 nm for dTTTTTT-Σ-dAAAAAA (compound **5**), which adopts a double stranded conformation, is small. It is also significant that the hyperchromicity determined at 260 nm is as great for compound **7** as for compound **5**. Since a dT_6/dA_6 double-strand segment is common to both compounds, this result indicates that both of the dT_6 segments in **7** contribute to the hyperchromic change and are therefore in a base-stacked conformation.

Introduction of dC/dG base pairs affords even more stable structures. A linear increase in absorbance (18% hyperchro-

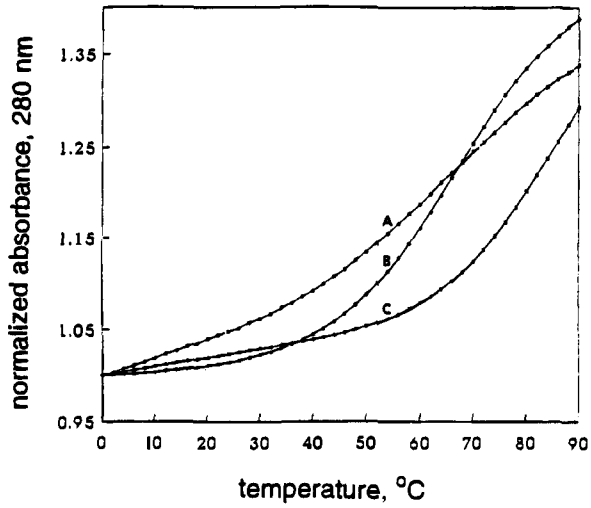


Figure 3. Thermal dissociation profiles: A, dGC-Σ-dGC, and C, dGCG-Σ-dCGC, in standard buffer; and B, dGCG-Σ-dCGC in 50% EtOH/H_2O (no added salt).

micity at 280 nm) was observed on heating dG-Σ-dC from 0 to 80 °C in 0.1 M NaCl solution, indicative of some degree of organization of the bases in this dinucleotide derivative at the lower temperatures. In contrast, the absorbance of dG/dC decreased slightly (2%) under the same conditions. The melting curve for dGC-Σ-dGC (see Figure 3) shows extensive base stacking for this small oligomer. In the case of dGCG-Σ-CGC, the melting curve exhibits a single, relatively sharp break; the base-stacked form of this oligonucleotide conjugate is so stable in aqueous 0.1 M NaCl that only the first break in the curve is observed over the range of 0−90 °C (T_m >80 °C; Figure 3). A sigmoidal curve is obtained when the solvent is changed to 50% ethanol/water with no salt. Even under these unfavorable conditions for hybridization, however, the base stacked form exhibits high stability (T_m 66 °C).

Binding of Small Molecules. To gain further information on the properties of these oligonucleotide conjugates, we examined the effects they have on the fluorescence of ethidium bromide and Hoechst 33258. In dilute solutions ethidium binds to double-stranded DNA by intercalation between base pairs,[19-21] approaching saturation as alternate base-pair pockets are filled. Hoechst 33258 binds laterally in the minor groove of double-stranded DNA at sequences containing three to five contiguous dT-dA pairs.[22-26] In both cases the interaction leads to strong enhancement of fluorescence of the dye. Single-stranded polynucleotides have little effect on the fluorescence of either dye.

All the self-complementary conjugates except dT-Σ-dA and dG-Σ-dC markedly increased the intensity of fluorescence of

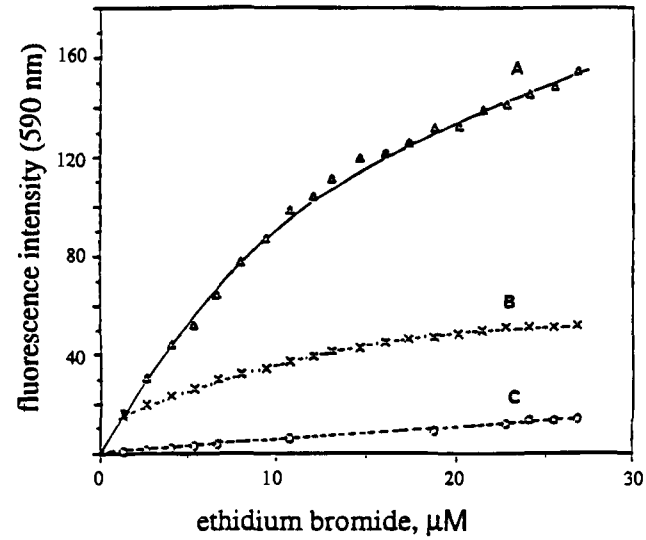


Figure 4. Fluorescence of ethidium bromide in presence of the following: A, dTT-Σ-dAA (6.5 μM); B, poly(dT)/poly(dA) (32 μM in dT and dA); or C, buffer without any nucleotides. The titrations were carried out by adding 4 μL aliquots of 1.0 mM ethidium bromide in water to the nucleotides in 3 mL of H_2O, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.0) at 5 °C. The solid line for A represents a calculated curve, [27] and the triangles, experimental data.

ethidium bromide. Data for titration of the smallest and least promising member of the dT/dA series, dTT-Σ-dAA, with ethidium bromide are given in Figure 4. A striking feature is the magnitude of the enhancement of fluorescence observed. Over most of the range of ethidium bromide concentrations, the enhancement at 5 °C is several times greater than that caused by poly(dT)/poly(dA). The data points for dTT-Σ-dAA agree well with a curve calculated on the basis that the oligomer and ethidium form a 1/1 complex, the association constant is 1 × 10^5 M^{-1}, and the ratio for the fluorescence of bound to unbound ethidium is 60.[27] The points for titration of poly(dT)/poly(dA) do not follow a simple curve. The enhancement caused by the initial aliquot of the polynucleotides is the same as that caused by dTT-Σ-dAA at a comparable nucleotide concentration, but the fluorescence increases are attenuated as the titration proceeds, suggesting some degree of self-quenching by ethidium intercalated in nearby pockets in the polymer. An increase in temperature from 5 to 25 °C has little effect on fluorescence in the poly(dT)/poly(dA)−ethidium system but leads to 64% reduction in enhancement in the dTT-Σ-dAA−ethidium system, reflecting the decrease in the fraction of the small oligomer in a base-stacked conformation at the higher temperature.

The fluorescence of Hoechst 33258 was also strongly enhanced by dTTTT-Σ-dAAAA and the longer bridged oligomers (see Figure 5). dTTT-Σ-dAAA had a weak but significant effect and dTT-Σ-dAA had no detectable effect on fluorescence

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(27) Points for the calculated curve for the fluorescence of ethidium in the presence of dTT-Σ-dAA (Figure 4, solid line) were derived using the equations $F_t = F_f + F_b$, $F_f = a(E_0 - E_b)$, and $F_b = baE_b$, where F_t, F_f, and F_b represent the total fluorescence, the fluorescence of free ethidium, and the fluorescence of bound ethidium, respectively, at a given total ethidium concentration, E_0. The concentrations of bound ethidium, E_b, and free ethidium, $E_0 - E_b$, were determined using the equilibrium expression, $K = E_b/(N_0 - E_b)(E_0 - E_b)$, with N_0, the concentration of dTT-Σ-dAA, equal to 6.5 μM and K taken as 1 × 10^5 M^{-1}. Constant a was obtained from a plot of fluorescence of ethidium as a function of concentration for experiments carried out under the same conditions in the same apparatus but in absence of the oligonucleotide (line C in Figure 4). The points fit the following equation: fluorescence = a(ethidium concentration), with a = 5.33 × 10^5 fluorescence intensity units/M. K and b were treated as adjustable parameters. A good fit to the experimental data was obtained with K = 1 × 10^5 M^{-1} and b = 60. b represents the enhancement in fluorescence associated with binding of ethidium to the oligonucleotide.

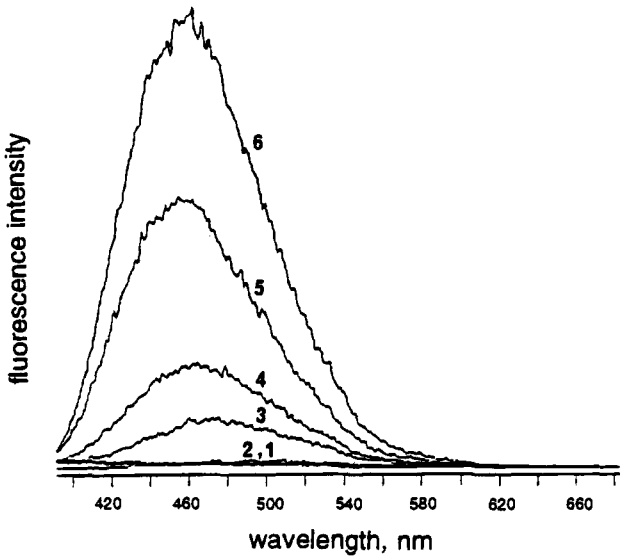

Figure 5. Fluorescence spectra (λ_{exc} 375 nm) for Hoechst 33258 (2.7 μM) in standard buffer (3 mL) at 23 °C in presence of 0.67 A_{260} units of oligonucleotide conjugates: top to bottom for compounds **6**, **5**, **4**, **3**, respectively, and at base line for compound **2**, **1**, or no added nucleotide.

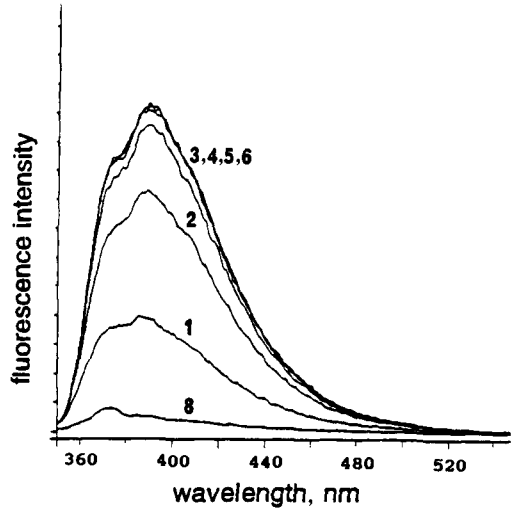

Figure 6. Fluorescence spectra in standard buffer at 23 °C for 0.1 mM oligomer solutions: top to bottom for compounds **3**–**6**, **2**, **1**, and **8**, respectively.

of this chromophore. These results suggest some similarity in the sites in these mini-duplex structures that bind Hoechst 33258 and the minor groove binding sites in conventional double-stranded oligonucleotides and DNA.

Fluorescence Spectra. Fluorescence spectra for representative stilbene–oligonucleotide conjugates (**1**–**6**) are shown in Figure 6. The presence of a strong band in the 386 nm region and absence of a band in the 445 nm region for the dT/dA derivatives show the absence of complexes that bring two stilbenedicarboxamide units close in space.[17] The spectra for compounds **7**, **8**, **12**, and **13** are similar in that only the 386 nm band is observed. In the case of dG/dC compounds **9**–**11**, quenching of fluorescence is so extensive that this structural probe is not applicable.

Another interesting feature of the fluorescence spectra is the pattern of the intensities of the dT/dA compounds. The intensity is least for compound **1**, which exists as an open "random coil", and is greatest for the longer oligomers, **3**–**6**, which are present primarily as organized, base-stacked structures. The lesser quenching of fluorescence for the longer oligomers probably stems from a more hydrophobic environment for the stilbene-carboxamide chromophore, which lies above the bases in the hybridized compounds. Consistent with this view, the intensity of fluorescence of oligomer **2**, which is partially in the organized and disorganized states, is intermediate between that for **1** and for **3**–**6**. The fact that the spectra for **3**–**6** are the same within the accuracy of the experiments indicates that adenine bases further removed than the third position from the bridge in the organized systems play no significant role in quenching fluorescence of the stilbene moiety.

The fluorescence intensity for these oligomers is greater in 50% ethanol/water than in water, in accord with the change in hydrophobicity of the solvent. For example, the enhancement factor is 4.5 for dT-Σ-dA and 1.4 for dTTTTTT-Σ-dAAAAAA. In 50% ethanol/water at 23 °C the relative intensities at λ_{max} for equimolar concentrations of diol II, dT-Σ-dA, dTTTTTT-Σ-dAAAAAA, dG-Σ-dC, and dGC-S dGC are 1.0, 0.9, 0.7. 0.2, and 0.05, respectively; no quenching relative to diol II is observed for dTTTTTT-Σ-dTTTTTT or dCCCCCC-Σ-CCCCCC. All these compounds are largely or completely in the random coil conformations in this solvent at this temperature, so complications arising from the presence of molecules in two states (base stacked and random coil) are minimized. One may therefore conclude that dT and dC are not effective quenchers of fluorescence of the stilbenecarboxamide chromophore, dA is a weak quencher, and dG is a strong quencher. This order correlates with the oxidation potentials for the purine and pyrimidine bases,[28] suggesting that quenching of fluorescence may proceed by electron transfer from a dG or dA base to the stilbene group.

Photoisomerization. Like stilbene,[29–31] *trans*-diol II was found to isomerize to the *cis* isomer on irradiation, reaching a photostationary state in 20% ethanol/water consisting of ~80% *cis* isomer. The dT/dA conjugates containing the stilbene chromophore react similarly, but somewhat less efficiently. Irradiation (>300 nm) of dTTTT-Σ-dAAAA in water afforded a mixture containing the *trans* isomer (~50%), a major photoproduct (~40%), and four minor products eluting earlier on RP HPLC. Similar results were obtained from a larger scale reaction of dTTTTTT-Σ-dAAAAAA. The T_m value for the major photoproduct isolated from the latter reaction by RP HPLC is 49 °C (Table 1), 10 °C lower than that for the *trans* isomer. We tentatively designate the major new product in each case as the *cis* isomer by analogy to the many examples of photoisomerism observed for related substituted stilbenes.[29–31,17] The lowering in the T_m value on irradiation of dTTTTTT-Σ-dAAAAAA may be attributed to shortening the length of the internucleotide bridge by conversion of the *trans*-stilbenedicarboxamide group to the *cis* isomer.

In contrast to the dT/dA derivatives, dGCG-Σ-dCGC and dTTGC-Σ-dGCAA proved to be stable to light >300 nm. No change in the UV spectrum or in the RP HPLC profile was observed for samples irradiated for long time periods (~30 times the period to achieve the photostationary state for diol II). This stability correlates with the quenching of fluorescence observed in oligomers containing dG/dC bases near the stilbenedicarboxamide chromophore.

Discussion

In principle, these oligonucleotide–stilbenedicarboxamide conjugates can give hypochromic structures either by folding

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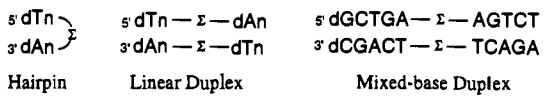
Chart 2



unimolecularly to a hairpin structure or by associating to a linear duplex (Chart 2). By analogy to oligonucleotide derivatives containing a terephthalamide linker,[11] one would expect the hairpin form to predominate. Data from several sources provide convincing evidence that this is indeed the case: (a) The T_m values for three representative oligomers, *trans*-dTTTTTT-Σ-AAAAAA, *cis*-dTTTTTT-Σ-dAAAAAA, and dT_{12}-Σ-dA_{12} (all in water) and dGCG-Σ-dCGC (in 50% ethanol/water) are independent of concentration over the 5-fold range examined. This result is typical for the melting transition of an oligonucleotide folded in a hairpin structure. (b) As demonstrated in previous work with -Σ- bridged, mixed-base oligomers that form linear duplexes,[17] complexes that bring stilbenecarboxamide groups in proximity in an aqueous environment exhibit a strong excimer fluorescence band at 445 nm and at best weak fluorescence in the 386 nm region characteristic for the stilbene chromophore. One may therefore conclude from the absence of a 445 nm band and presence of a strong band near 386 nm in the spectra of all the fluorescent compounds represented in Figure 6 that conformations that place two stilbene groups in proximity are unimportant in this system. The fluorescence data therefore exclude as significant structures linear duplexes and also bimolecular complexes that might form by hydrophobic association of two stilbene groups contained in separate hairpin conformers. (c) The high transition temperatures for these dT/dA conjugates are also inconsistent with formation of linear duplexes. Thus, the mixed base duplex formed from the oligonucleotide conjugates, 5'dGCTGA-Σ-dAGTCT/3'dCGACT-Σ-dTCAGA (Tm 37 °C), is somewhat less stable than the corresponding oligonucleotide duplex lacking the bridges, 5'dGCTGAAGTCT/3'dCGACTTCAGA (T_m 41 °C[17]). By analogy, one would expect dTTTTTTAAAAAA, which forms a linear duplex,[11] to be more stable than dTTTTTT-Σ-dAAAAAA if the latter were also in the linear duplex state. In fact, however, the reverse is true; the T_m value for the stilbenedicarboxamide conjugate (59 °C) is 41 °C higher than that for dTTTTTTAAAAAA (18 °C[11]).

Unusual thermal stability was observed for (dTTTTTT-Σ-)$_2$dAAAAAA (**7**), as well as for the oligomers that form a simple hairpin structure. The high hyperchromicity exhibited in the melting curves of **7** monitored at both 280 and 260 nm is indicative of dissociation of a triple-stranded complex. Two different triple-stranded structures might form. One is a doubly folded, unimolecular conformer, and the other is a bimolecular complex in which an oligo(dA) segment of each molecule is bound to both oligo(dT) segments of the other. The fluorescence properties of the stilbenedicarboxamide bridge enable one to distinguish between these structures. Appearance of a band at 386 nm and absence of a long wavelength band rule out a bimolecular complex, which would place two stilbene chromophores in proximity and give rise to excimer emission.[17] We conclude that compound **7** adopts a doubly folded, mini-triple-stranded conformation.

The interaction of the intercalating agent ethidium bromide and the groove binding dye Hoechst 33258 with these small hairpin oligonucleotides resembles the behavior of these dyes with DNA. Our experiments show that an oligomer with a mini-groove formed by as few as three base pairs can bind Hoechst 33258 and that one with only two base pairs can bind ethidium efficiently in dilute solution.

In summary, these results show that the stilbenedicarboxamide linker is a unique and exceptionally effective cap for stabilizing double- and triple-stranded conformations of oligonucleotides. Constructs containing complementary oligonucleotides linked by a stilbenedicarboxamide bridge are attractive candidates as small molecule models of segments of DNA. They may also prove useful as replacements for extended double-stranded stem regions that stabilize unusual secondary and tertiary conformations in RNA and as structural components in designing novel self-assembling systems. Several features of the chemistry of the conjugates are noteworthy. (a) The dissociation temperatures for the unimolecular complexes are unusually high. To our knowledge the thermal stability of the small, double- and triple-stranded oligonucleotide domains that can be formed is without precedence. We attribute the effectiveness to a favorable geometry of the overall bridge, which can fit across the top of an oligonucleotide duplex, and to the presence of an extended, relatively rigid aromatic core that reduces the degrees of freedom in the linker. (b) The fluorescence spectrum of the stilbene moiety provides a means for monitoring the local environment of the bridge. In particular, it enables one to distinguish between organized assemblies that bring two stilbene units into proximity (e.g., as in the mixed base duplex in Chart 2 that exhibits excimer fluorescence) and structures containing isolated stilbenedicarboxamide groups (e.g. as in the monomolecular hairpins which fluoresce like stilbene). (c) The stilbenedicarboxamide group serves as a vehicle for photoinducing a change in the dimensions of the bridge and therefore, altering the hybridization properties of the oligonucleotide conjugate. (d) Finally, the sensitivity of an oligonucleotide conjugate to light can be controlled by design of the sequence of nucleotides adjacent to the bridging element. When connected to nucleotide sequences starting with dG/dC pairs, the stilbenedicarboxamide bridge is highly resistant to photoinduced reactions; when connected directly to dT/dA pairs, it is sensitive to light.

Experimental Section

General Methods. Reagent grade chemicals were used throughout. Pyridine, DMF, and acetonitrile were dried over calcium hydride. THF was freshly distilled from sodium prior to use. *trans*-Stilbene-4,4'-dicarboxylic acid was purchased from Lancaster, NH. Column chromatography was performed on silica gel (70−230 mesh, 60 A). TLC was carried out on Whatman analytical silica gel plate (60A). Reversed phase (RP) HPLC was carried out on a Dionex chromatograph with a Hewlett Packard Hypersil ODS-5 column (4.6 × 200 mm) and a 1%/min gradient of acetonitrile in 0.03 M triethylammonium acetate buffer at pH 7.0 with a flow rate of 1 mL/min. A Dionex NucleoPac PA-100 column (4 × 250 mm) was used for ion exchange (IE) chromatography. The eluant was 10 mM aqueous NaOH, and a 2%/min gradient of 1.5 M NaCl in 10 mM NaOH was used; flow rate of 1.0 mL/min. NMR spectra were run on a Varian XL400 instrument in $CDCl_3$ with TMS as the internal reference for proton spectra and 85% H_3PO_4 as an external reference for ^{31}P spectra.

Melting curves were determined using a Perkin Elmer Lambda 2 UV spectrophotometer equipped with a temperature programmer for automatically increasing the temperature at the rate of 0.8 °C/min. Denaturation was followed by the absorbances at 260 nm for most oligomers and by the absorbance at 280 nm for conjugates containing only dC/dG nucleotides. Fluorescence spectra were obtained on a Perkin Elmer LS 50B spectrofluorimeter equipped with a RM6 Lauda circulating bath for controlling the temperature. Except where otherwise noted, the dissociation and fluorescence experiments were carried out in aqueous solutions 0.1 M in NaCl and 10 mM in Tris-HCl at pH 7.0 (designated as standard buffer) with a nucleotide concentration of 1.0 A_{260} units/mL for the T_m measurements and 0.02 A_{334} units/3 mL for the fluorescence studies.

Diol II. A suspension of stilbene-4,4'-dicarboxylic acid (2.3 g; IR C=O, 1750 cm^{-1}) in benzene (40 mL), dimethylformamide (0.1 mL),

and thionyl chloride (1.4 mL) was refluxed overnight. Removal of the solvents at reduced pressure afforded the acid chloride as a yellowish solid (2.8 g; IR C=O, 1695 cm^{-1}). Tetrahydrofuran (20 mL) was added and the resulting slurry was slowly poured into a well-stirred solution of 3-aminopropanol (1.52 mL, 2.2 equiv) and triethylamine (6.26 mL, 5 equiv) in methanol (40 mL) at 0 °C. After being warmed to room temperature, the mixture was concentrated to ~20 mL and poured into water (200 mL). The solid was collected and washed successively with methanol to give 3.0 g (87%) of diol II: mp 299–301 °C; IR C=O 1629 cm^{-1}; UV (50% ethanol/water) λ_{max} 320, 330, 344 nm; FAB MS M + 1, 383; ^{1}H NMR (DMSO-d_6, ppm) 8.50 (t, 2NH), 7.89 (d, J_{H-H} = 8.5 Hz, 2H2, H2′ of stilbene), 7.71 (d, J_{H-H} = 8.5 Hz, 2H3, H3′ of stilbene), 7.42 (s, −CH=CH−), 4.51 (t, 2OH), 3.48 (q, $2CH_2O$), 3.32 (q, $2CH_2N$), 1.70 (m, $2CH_2$).

Mono(dimethoxytrityl) Derivative of Diol II (Compound III). Compound II (1.5 g) was coevaporated twice with dry pyridine and suspended in a mixture of dimethylformamide (70 mL) and pyridine (15 mL). The mixture was warmed to ~70 °C to dissolve diol II, dimethoxytrityl chloride (1.3 g) was added, and the solution was stirred as the mixture cooled to room temperature. After 2 h, analysis by TLC indicated the presence of about equal amounts of diol II, the mono-DMT derivative (compound III), and the bis-DMT derivative. Additional dimethoxytrityl chloride (0.7 g) was added, and after stirring for an additional 1.5 h, the mixture was poured into saturated aqueous sodium bicarbonate. The precipitate was collected, washed with water and ether, and taken up in 200 mL of methanol/chloroform (1/9). After filtration to remove residual diol II, the solution was concentrated and the products were dissolved in chloroform and separated by chromatography on silica gel (eluant, 2–4% methanol in chloroform). The faster moving compound corresponded to the bis(dimethoxytrityl) derivative (R_f 0.9; 1 g, 20%). The slower moving material was compound III (R_f 0.3; 1.4 g, 52%): mp ~140 °C with decomposition; UV ($CHCl_3$), λ_{max} 320, 330, 348 nm; ^{1}H NMR ($CDCl_3$, ppm) 7.79 and 7.80 (s, 2NH), 7.56 and 7.58 (d, 2H2, H2′ of stilbene) 7.40–7.45 (m, 2H3, H3′ of stilbene), 7.16–7.35 (m, −CH=CH− and DMT), 6.81 and 6.84 (s, H3 and H5 of anisyl), 3.78 (s, $2CH_3O$), 3.28–3.76 (m, $2NHCH_2$ and $2CH_2OH$), 1.81–1.90 and 1.92–1.94 (m, $-CH_2-$ and $-CH_2-$). Anal. Calcd for $C_{44}H_{44}N_2O_4 \cdot H_2O$: C, 72.70; H, 6.33; N, 3.86. Found: C, 72.95; H, 6.32; N, 4.09.

Preparation of Phosphoramidite Derivative IV. Chloro(diisopropylamino)(β-cyanoethoxy)phosphine (0.23 mL, 1.0 mmol) was added with stirring to a solution of compound III (0.63 g, 1 mmol) and diisopropylethylamine (0.63 mL, 3.6 mmol) in dry tetrahydrofuran (10 mL). After 3 h, the solution was poured into ethyl acetate (200 mL, prewashed with 5% sodium bicarbonate) and extracted with 5% sodium bicarbonate. The organic layer was dried with sodium sulfate and concentrated, and the phosphitilated product was isolated by chromatography on silica gel (eluant, 2% triethylamine in ethyl acetate). Fractions containing compound IV were concentrated and precipitated in cold pentane at −70 °C to give 0.5 g (62%) of IV as a white powder: R_f 0.8 (ethyl acetate containing 1% triethylamine); UV ($CHCl_3$), λ_{max} (nm) 320, 330, 348; ^{1}H NMR ($CDCl_3$, ppm) 7.81 and 7.83 (s, 2NH), 7.57 and 7.61 (m, 2H2, H2′ of stilbene), 7.42–7.44 (m, 2H3, H3′ of stilbene), 7.18–7.34 (m, −CH=CH− and DMT), 6.81 and 6.84 (s, H3 and H5 of anisyl), 3.79 (s, $2CH_3O$), 3.32–3.35, 3.55–3.77, and 3.81–3.90 (m, $2NHCH_2$ and $2CH_2ODMT$ and $POCH_2CH_2CN$), 2.64 (t, 2CH of 2 iPr), 1.90–1.99 (m, $-CH_2-$ and $-CH_2-$), 1.16–1.29 (m, 4 Me of 2iPr); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as the external reference) 146.3 ppm.

Oligonucleotides. Oligonucleotide–stilbenedicarboxamide conjugates were synthesized by conventional phosphoramidite chemistry using cyanoethyl phosphoramidite reagents and compound IV in a Milligen/Biosearch Cyclone synthesizer or a Millipore Expedite oligonucleotide synthesizer. The standard procedure was followed in the assembly and deprotection of the oligomers. The coupling yield in the step utilizing IV was satisfactory (>95%). The oligomers were isolated as trityl-on derivatives by RP HPLC and then detritylated with 80% acetic acid (30 min). Both IE (pH 12) and RP HPLC showed that samples of oligomers prepared in this manner were satisfactory (>95%). The oligomer containing a tris(ethylene glycol) bridge was prepared by the same procedure used for the stilbenedicarboxamide derivatives, except dimethoxytrityl-$(OCH_2CH_2O)_3P(OCH_2CH_2CN)$-$(NiPr_2)$ (Glen Research, VA) was used in place of compound IV. Concentrations of the oligonucleotide–stilbenedicarboxamide conjugates were calculated on the basis of the absorbance of the stilbenedicaboxamide chromophore, using ϵ_{334} = 37 L/mmol/cm. The concentrations of nucleotide bases in solutions of poly(dT) and poly(dA) were calculated using ϵ_{260} = 8.1 L/mmol/cm for dT and 8.4 L/mmol/cm for dA units.[18]

Photochemistry. Samples of dT/dA conjugates in dilute solutions were found to isomerize rapidly in bright sunlight. For example, when a solution of *trans*-dTTTTTT-Σ-dAAAAAA (3 A_{260} units in 1 mL of water) was deaerated by bubbling with nitrogen for 5 min and then exposed to sunlight through a glass pane for 8 min, the absorbance at 336 nm (stilbene chromophore) decreased while that at 260 nm (nucleotide bases) was essentially unchanged. RP HPLC analysis showed two sharp, major peaks at 18.1 min (photoproduct) and 18.7 min (oligomer **5**) and several minor peaks at 15.5–17 min. Isolation of the major peaks afforded 1.4 A_{260} units of the photoproduct (*cis* isomer) and 1.0 A_{260} units of oligomer **5** (*trans* isomer). The UV spectrum in the long wavelength region for this photoproduct (λ_{max} 316 nm) corresponds to that for *cis*-stilbene. This reaction is similar to that observed on irradiation diol II and individual mixed-base oligonucleotide stilbenedicarboxamide conjugates.[17] In the diffuse light of the laboratory, the oligonucleotide conjugates are reasonably stable, so they can be handled without difficulty. For extended storage it is advisable to keep them in the dark as refrigerated solutions or lyophilized solids.

A Rayonet reactor equipped with two Rayonet lamps (350 nm, 24 W) situated on opposite sides of the reactor was used to investigate the photoreactions further. The sample to be irradiated was placed in the center of the reactor about 10 cm from the lamps. Thus, a solution of diol II (0.25 A_{328} units) in ethanol/water (20/80; 1.0 mL) in a UV cuvette was irradiated in the Rayonet reactor for 30, 60, and 90 s. During this time, the absorbance at 328 nm decreased to one-third of its initial value. No significant change was observed on longer irradiation. Periodic analysis of aliquots (100 μL) by RP HPLC, using a gradient of 20%-40% acetonitrile in 0.1 M triethylammonium acetate (pH 7), showed conversion of the *trans* isomer (elution time of 10.5 min) to a mixture containing the *trans* and *cis* (elution time of 13.5 min) isomers in a 1/4 ratio.

Reactions of the oligonucleotide conjugates were carried out similarly except that the solvent was 0.1 M NaCl and 10 mM Tris-HCl buffer (pH 7). For example, irradiation of compound **4** (0.25 A_{334} units) in 1.0 mL of buffer was followed by changes in the UV spectrum and RP HPLC profile. The absorbance at 334 nm for the stilbene chromophore fell progressively from 0.25 to 0.15, 0.14, and 0.11 A_{334} units after irradiation times of 5, 10, and 20 min. RP HPLC analyses revealed partial conversion of *trans*-TTTT-Σ-AAAA (retention time of 21.3 min) to a new product (21.6 min) along with minor components eluting in the 18.3–20 min range. After 10 min of irradiation the mixture contained approximately 50% *trans* isomer, 40% of the major photoproduct, and 10% of the minor products, as judged by the HPLC profiles. Longer exposure times (to 20 min) did not change the ratio of the two isomers but led to more of the minor products.

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