Hybrid Oligonucleotides Containing Stilbene Units. Excimer Fluorescence and Photodimerization

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Abstract: Complementary pairs of oligonucleotides in which the stilbene chromophore is incorporated either in the middle or at the end of a 10 base pair sequence have been prepared and their spectroscopic and photochemical properties investigated. The individual oligonucleotides display fluorescence spectra and photoisomerization similar to that of a model 4,4′-stilbenedicarboxamide. Variations in fluorescence quantum yield and decay times are attributed to interactions with neighboring bases. One-to-one mixtures of complementary oligonucleotides form stable duplexes with melting temperatures of 40 and 46 °C, respectively, for the mid-strand and terminally labeled duplexes. Duplex formation results in hyperchromism of both the nucleotide and stilbene absorption bands and the appearance of broad, long-wavelength fluorescence attributed to an excited stilbene dimer. These duplexes provide a unique opportunity for the investigation of the here-to-for elusive stilbene excimer. The long wavelength absorption bands of both duplexes are efficiently bleached upon irradiation. Bleaching is a consequence of stereospecific stilbene [2+2] photodimerization and results in the formation of cross-linked duplexes of high thermal stability. Excimer and monomer fluorescence are found to provide a highly sensitive probe of duplex formation and dissociation.

Introduction

Hybridization of complementary oligonucleotides results in precise alignment of hydrogen-bonded base pairs.[1] Investigations from several laboratories have established that the formation of duplex structures is tolerant of the incorporation of nucleosides with "unnatural" bases[2] and polyether[3] or aromatic dicarboxamide[4] spacers. One of our laboratories has recently reported the formation of a duplex between two complementary oligonucleotides consisting of two 5 base pair segments connected by a stilbenedicarboxamide (**1:2**).[5] Duplex formation was found to have a profound effect upon the photochemical behavior of the stilbene chromophore.[6] The normal photochemical behavior of the stilbene chromophore in the individual oligomers (stilbene fluorescence and photoisomerization) is replaced by behavior attributed to the stilbene excimer (excimer fluorescence and photodimerization) in the duplex. Since stilbene excimer fluorescence is not observed upon self-quenching of singlet stilbene in organic solution[7] and has only been observed for a few covalently bonded stilbenes,[8–10] duplex formation provides a unique opportunity to investigate the photochemical behavior of the stilbene excimer. Thus we have undertaken a collaborative investigation of the photophysical and photochemical behavior of this system and of the related duplex **3:4**, in which the stilbene chromophores are positioned at the end of a complementary 10-base pair sequence. We find that excimer and locally excited fluorescence provide a highly sensitive probe of duplex formation and dissociation and that stilbene photodimerization results in the formation of crossed-linked duplexes of high thermal stability.

Results

Synthesis and Absorption Spectra. *trans*-Stilbene-4,4′-dicarboxylic acid was converted to the *N,N′*-dimethylamide **5a** and the *N,N′*-(3-hydroxypropyl)amide **5b** via the reaction of the diacid chloride with methylamine or 3-amino-1-propanol. Diol **5b** was converted to the phosporamidite reagent and the oligomers **1**–**4** were synthesized using standard phosphoramidite chemistry. Detailed synthetic procedures are reported elsewhere.[11] Solutions of the oligomers are light sensitive, but are stable for periods of several weeks in the dark. Lyophilized samples can be stored for extended periods and reconstituted by addition of water without apparent change in their spectroscopic properties.

The absorption spectra of oligomers **1** and **2** at 23 °C in aqueous 0.1 M NaCl, pH 7.0 (10 mM Tris·HCl), are shown in Figure 1. The absorption spectra of oligomers **3** and **4** are

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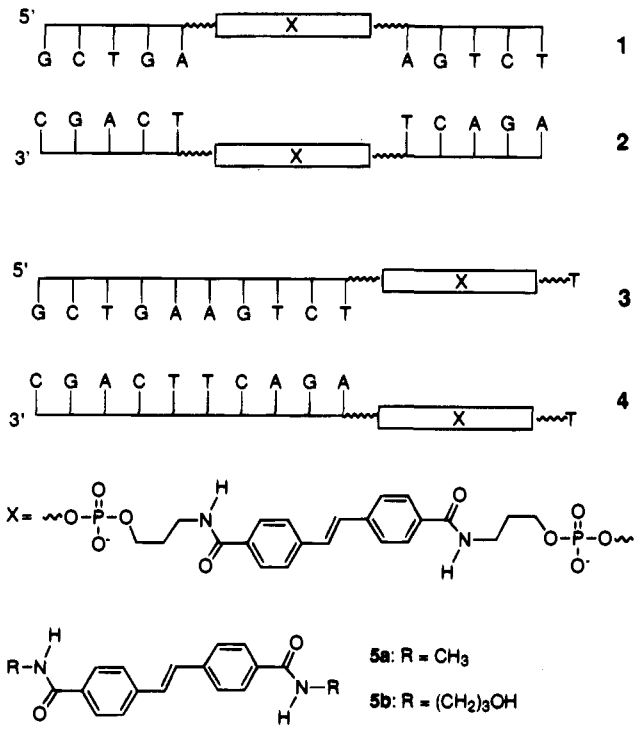
Chart 1



similar to those of **1** and **2**. All four oligomers display a weakly structured long-wavelength absorption band (λ_{max} = 336 nm) similar to that of the stilbene diol **5b** and a short-wavelength structureless band (λ_{max} = 260 nm) dominated by the nucleotide bases. The spectrum of a 1:1 mixture of **1** and **2** (**1:2**) is also shown in Figure 1. The long-wavelength band for **1:2** is broader than that of the oligomers and its maximum is at higher energy (330 nm). Hyperchromicity is observed for both absorption bands when a solution of the complex is heated. Melting curves for the duplex **1:2** obtained from the 260 and 330 nm absorption data are shown in Figure 2. Melting temperatures and hyperchromicities obtained from the 260 and 330 nm absorption data for both duplexes and for a duplex contaiing the same sequence of 10 base pairs present in **1:2** but lacking the bridging stilbene elements are reported in Table 1.

Fluorescence Spectra. The diol **5b** displays moderately intense, weakly structured fluorescence in aqueous solution (Figure 3a). The fluorescence quantum yield is temperature dependent, decreasing from 0.17 at 0 °C to 0.06 at 50 °C. The fluorescence decay of **5b**, as determined using time correlated single photon counting, is best fit to a single exponential with a decay time of 0.39 ns at 0 °C and 0.28 ns at 23 °C. The oligomers **1**−**4** all display fluorescence in the same spectral region as that of **5b** (Figure 3a,b), however their fluorescence quantum yields in aqueous solution are lower than that of **5b** (Table 2). The shoulder observed at ca. 370 nm in the oligomer fluorescence spectra is an instrumental artifact. The fluorescence quantum yield of oligomer **4** is temperature dependent, decreasing from 0.08 at 0 °C to 0.05 at 50 °C. The fluorescence decay of the oligomers cannot be fit to a single exponential and is best fit by either a dual (**2** and **3**) or triple (**1** and **4**) exponential. A decaying component with a lifetime longer than that of **5b** is observed for all four oligonucleotides. Decay times measured at 2 °C are reported in Table 2.

Also shown in Figure 3 are the fluorescence spectra of 1:1 mixtures of **1** and **2** and of **3** and **4** at 0 °C. These spectra are dominated by broad structureless emission which is attributed to the stilbene excimer. Fluorescence spectra observed upon titration of **1** with 0.2 equiv aliquots of **2** are shown in Figure 4. Titration of **1** with **2** results in an increase in excimer

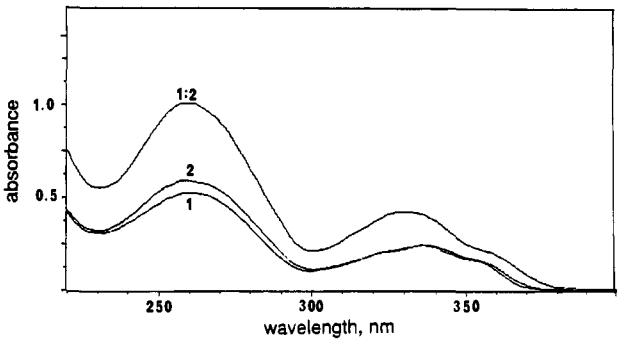


Figure 1. Ultraviolet absorption spectra of stilbene-containing oligomers **1** and **2** and the duplex **1:2** at 23 °C in aqueous solution (0.1 M NaCl and 0.1 mM Tris·HCl buffer, pH = 7.0).

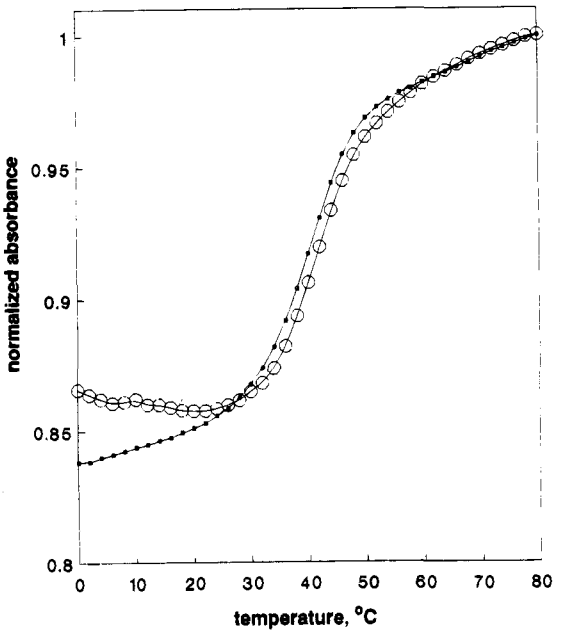


Figure 2. Melting curves for the duplex **1:2** monitored at 260 nm (■) and 330 nm (○).

Table 1. Melting Temperatures and Hyperchromicities for the Stilbene-Containing Duplexes

duplex[a]	260 nm, T_m, °C	260 nm, h^b	330 nm, T_m, °C	330 nm, h^b
reference[c]	41	0.18		
1:2	39	0.15	41	0.14
3:4	47	0.18	48	0.11
irrad **1:2**	67			
irrad **3:4**	>80			

[a] Aqueous solutions 5 × 10^{-6} M in each oligonucleotide (1.0 A_{260} units), 0.1 M NaCl and 0.1 mM Tris·HCl buffer (pH 7). [b] $h = (A_{60} - A_{20}/A_{20})$ where subscripts are temperatures at which absorbance is measured. [c] Duplex lacking stilbene units.

fluorescence until the equivalence point is reached. Further addition of **2** results in an increase in the short-wavelength emission (presumably that of **2**) but no further increase in excimer emission. Similar results are observed for titration of **3** with **4**. The stilbene excimer fluorescence is rapidly bleached upon exposure to ultraviolet light, resulting in a decrease in the ratio of excimer:locally excited fluorescence. This necessitates the use of narrow excitation slits, rapid scan rates, and freshly prepared solutions in order to optimize the excimer fluorescence intensity and minimize locally excited stilbene fluorescence.

The temperature dependence of the fluorescence spectrum of the duplex **1:2** is shown in Figure 5. The excimer fluorescence intensity decreases continuously with increasing temperature, whereas the locally excited stilbene fluorescence

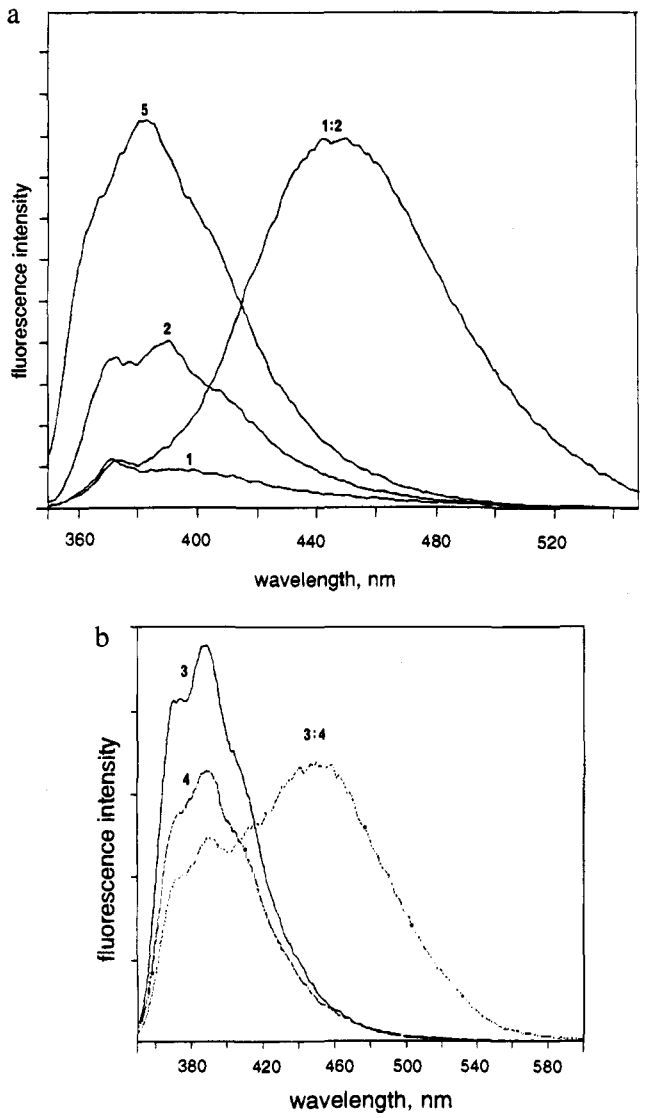


Figure 3. (a) Fluorescence spectra of model compound **5b** in aqueous solution and of oligomers **1** and **2** and duplex **1:2** at 0 °C in aqueous solution. (b) Fluorescence spectra of oligomers **3** and **4** and duplex **3:4** at 0 °C in aqueous solution (0.1 M NaCl and 0.1 mM Tris·HCl buffer).

Table 2. Quantum Yields and Fluorescence Decay Times for the Locally Excited Stilbene Fluorescence from Stilbene-Containing Oligomers and Duplexes

stilbene	Φ_f[a]	τ, ns (%)[b]
1	0.02	0.10 (39), 0.49 (52), 1.6 (9)
2	0.07	0.48 (12), 1.1 (88)
3	0.08	0.44 (46), 1.1 (54)
4	0.12	0.05 (21), 0.33 (50), 0.86 (29)
5b[c]	0.17 [0.11][c]	0.39 (100) [0.28][c]

[a] Quantum yield for 385 nm fluorescence in aqueous solution at 2 °C. [b] Decay times for 385 nm fluorescence in aqueous solution at 0 °C. Values in parentheses are contributions of the various decays to the total fluorescence intensity. [c] Values measured in 4:1 aqueous ethanol solution. Values in brackets determined at 23 °C.

intensity increases only at temperatures above 25 °C. These changes are fully reversible. Similar results are observed for the duplex **3:4**. Plots of the temperature dependence of the normalized locally excited fluorescence intensity for duplex **3:4** at two different concentrations (ca. 5×10^{-6} and 5×10^{-7} M in each oligonucleotide) are shown in Figure 6. Excimer fluorescence quantum yields (Φ_{fex}) and decay times for **1:2** and **3:4** at several temperatures are reported in Table 3. Values of Φ_{fex} for both duplexes decrease with increasing temperature.

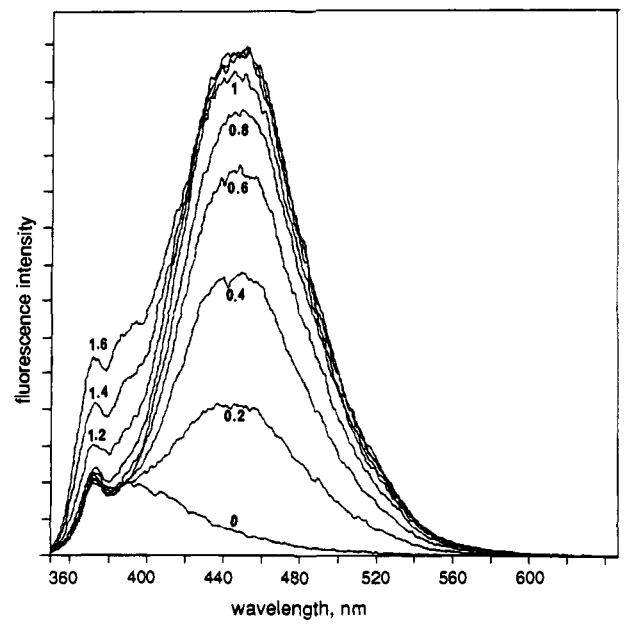


Figure 4. Fluorescence spectra for titration of an aqueous solution of oligomer **1** with 0.2 equiv aliquots of oligomer **2**.

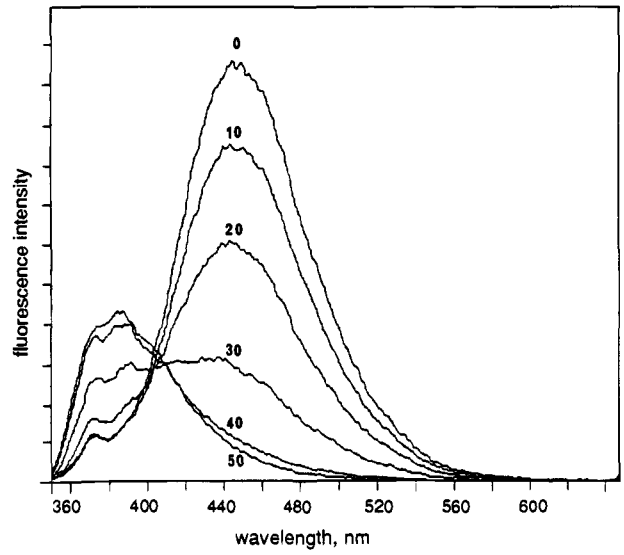


Figure 5. Temperature dependence of the fluorescence spectrum of the duplex **1:2**.

The excimer fluorescence decay of both duplexes can best be fit by a dual exponential with both decay times significantly longer than those for monomer emission from the oligomers or duplexes (Table 2). When the fluorescence decay is monitored at wavelengths shorter or longer than the emission maximum, no change in the decay times or preexponentials is observed. Neither the decay times nor the preexponentials change during data acquisition. Both the excimer fluorescence intensity and decay times are diminished in the presence of air or oxygen when compared to nitrogen-purged solutions.

Increasing the pH of **1:2** from 7 to 12 at 0 °C results in disappearance of the exciplex fluorescence and appearance of stilbene monomer fluorescence. Neutralization results in regeneration of the excimer fluorescence.

Photochemical Behavior. Continuous irradiation of the oligomers **1**−**4** and duplexes **1:2** and **3:4** with 350 nm light results in a decrease in the intensity of the 350 nm absorption band, but little change in the intensity of the 260 nm absorption band. Plots of A_{350} vs irradiation time for oligomer **2** and both duplexes are shown in Figure 7. The change in appearance of the 336 nm absorption band in **2** is similar to that for conversion

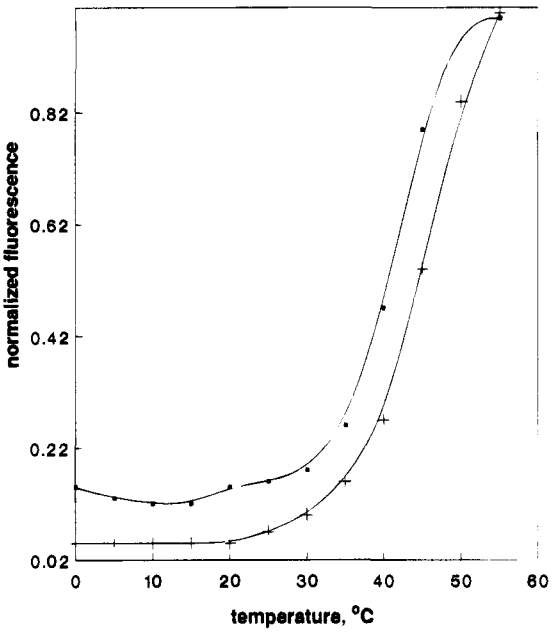


Figure 6. Temperature dependence of the normalized locally excited fluorescence intensity from the duplex **3:4** for oligonucleotide concentrations of 5×10^{-6} M (+) and 5×10^{-7} M (■).

Table 3. Quantum Yields and Fluorescence Decay Times for Stilbene Excimer Fluorescence from Stilbene-Containing Duplexes

duplex	temp, °C	Φ_{fex}[a]	τ_s, ns(%)[b]	τ_l, ns (%)[c]	Φ_d[d]
1:2	2	0.32	7.9 (41)	35.8 (59)	0.18[e]
	23	0.18	8.1 (45)	17.6 (55)	0.20 (0.34)
3:4	2	0.16	6.3 (36)	35.7 (64)	
	8	0.11	6.2 (32)	20.4 (68)	
	23	0.06	5.3 (57)	15.2 (43)	0.20

[a] Quantum yield for 455 nm fluorescence in nitrogen-purged aqueous solution. [b] Decay time of the short-lived component in nitrogen-purged aqueous solution monitored at 500 nm. Values in parentheses are contributions of the various decays to the total fluorescence intensity. [c] Decay time of the long-lived component in nitrogen-purged aqueous solution monitored at 500 nm. Values in parentheses are contributions of the various decays to the total fluorescence intensity. [d] Quantum yield for disappearance of duplex in aerated solution using 300 nm light. Value in parentheses for N_2-purged solution. [e] Determined at 5 °C.

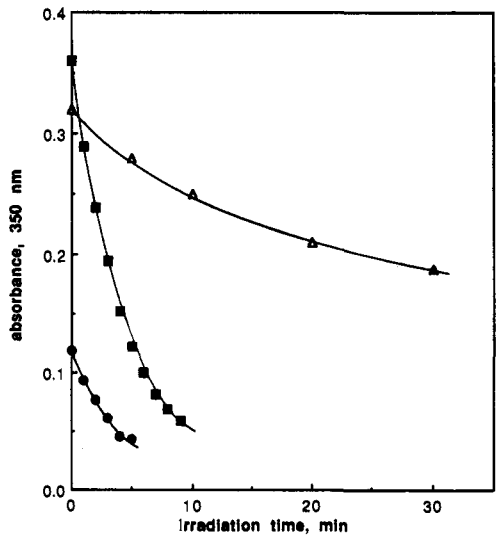


Figure 7. Bleaching of the 330 nm absorption band of the oligomer **2** (△) and the duplexes **1:2** (■) and **3:4** (●) upon irradiation at 300 nm.

of *trans*-stilbene to a photostationary state mixture of cis and trans isomers. Much more rapid bleaching of the 350 nm band is observed for the duplexes than for **2**. In addition, plots of

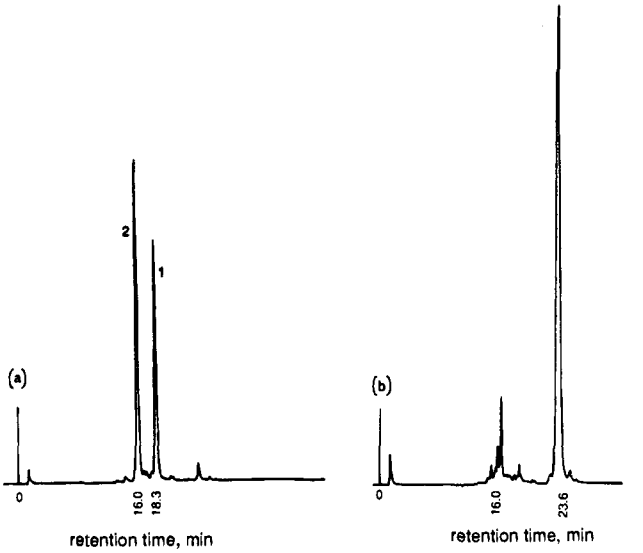
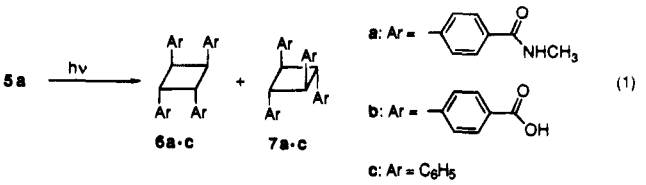


Figure 8. (a) Ion exchange HPLC traces for the duplex **1:2** prior to irradiation and (b) following irradiation.

$(\log A_{350})^{-1}$ vs irradiation time for the duplexes are linear to fairly high conversions, whereas the log plot for **2** is curved even at low conversions. Quantum yields for disappearance of the duplexes **1:2** and **3:4** upon irradiation with 330 nm light were determined both by the extent of bleaching of the 350 nm absorption band and by integration of the HPLC traces at moderate conversions (<20%) of reactants. Values for air-saturated or nitrogen-purged solutions are reported in Table 3.

The changes which occur upon irradiation of the oligomers and duplexes were also monitored by both ion exchange (IE) and reverse phase (RP) HPLC. In the case of the oligomers **1**–**4**, the major products observed at both low and high conversions have HPLC retention times similar to those of the reactants and are tentatively identified as the *cis*-stilbene-containing oligomers. At conversions of 50% or greater, additional products are observed. Irradiation of the duplex **1:2** or **3:4** results in the appearance of a product peak with significantly longer IE HPLC retention time than either reactant (Figure 8), suggesting the formation of a higher molecular weight species. Analysis of these solutions by RP HPLC shows the formation of a single product from **1:2** and the formation of two products with similar retention times from **3:4**. Minor peaks, corresponding to the major products of irradiation of the individual oligomers, are also observed upon irradiation of the duplexes. Melting temperatures for the duplexes **1:2** and **3:4** irradiated to high conversions are significantly higher than those obtained prior to irradiation (Table 1).

Irradiation of the stilbene diamide **5a** in dimethyl sulfoxide solution results in the formation of a mixture of the [2 + 2] photodimers **6a** and **7a** (eq 1). Alkaline hydrolysis followed



(1)

by acidification resulted in the precipitation of a mixture of the corresponding tetracarboxylic acids **6b** and **7b**. Dimer stereochemistry was assigned on the basis of comparison of the ^{1}H NMR spectra to those for the analogous *trans*-stilbene photodimers **6c** and **7c**.[7d] Irradiated samples of the oligonucleotide duplexes **1:2** or **3:4** were also subjected to alkaline hydrolysis (see Experimental Section). Comparison of the resulting

solutions with the mixture of **6b** and **7b** by RP HPLC indicated that only the plane-symmetric dimer **6b** was obtained from the irradiated duplexes.

Discussion

Duplex formation between stilbene-containing oligonucleotides provides a unique platform for investigating the ground state interaction and photochemical behavior of two stilbene chromophores in dilute solution. In addition, stilbene excimer fluorescence provides a novel and a potentially useful method for the investigation of duplex formation and dissociation. Stilbene photodimerization also provides a new method for cross-linking complementary pairs of oligomers. We will first discuss the photophysical and photochemical behavior of the stilbene-containing oligomers and duplexes. This behavior will then be compared to that for other stilbene-excimer-forming systems. Finally, applications to the investigation of duplex formation and photochemical cross-linking will be discussed.

Absorption Spectra. The stilbene-containing oligonucleotides **1**−**4** display long-wavelength (336 nm) absorption bands similar to those of the stilbenedicarboxamide **5b** and short-wavelength (258 nm) absorption bands similar to those of normal decanucleotides (Figure 1). Mixing the complementary stilbene-containing oligomers **1** and **2** or **3** and **4** at room temperature results in hypochromicity of both absorption bands. Broadening of the stilbene absorption band is observed in both duplexes, the band maximum shifting to shorter wavelength and the onset of absorption shifting to longer wavelength (Figure 1).

Hypochromicity in the 260 nm band is attributed to π-stacking of the nucleotide base pairs in the complementary nucleotide sequences of the duplexes **1:2** and **3:4**.[1] The hyperchromicities and melting temperatures (T_m) observed upon heating the stilbene-containing duplexes are similar to those for a duplex with the same sequence but lacking the stilbene chromophore (Table 1). The slightly lower value of T_m for the duplex **1:2** may reflect interruption of the π-stacking of the nucleotide base pairs in mid-strand by the stilbene fragments, whereas the slightly higher value of T_m for the duplex **3:4** may result from attractive interactions between the two terminal stilbene fragments. Similar results have been reported by Schweitzer and Kool[2c] for the insertion of hydrophobic, non-hydrogen-bonded isosteres of pyrimidines and purines in the middle and at the end of 12 base pair duplexes. Larger increases in T_m have been observed for a duplex formed between complementary oligonucleotides containing terminal cholesteryl substituents.[12]

Hypochromicity in the stilbene absorption band presumably results from π-stacking of the two stilbene chromophores. Inspection of molecular models indicates that the propylamide tethers used to incorporate the stilbene chromophore into the oligomers **1**−**4** are sufficiently long to permit $\pi-\pi$ interaction between the two stilbenes in the duplexes, when all the purines and pyrimidines are base paired. The hypochromicity of the stilbene absorption band in mid-strand duplex **1:2** is somewhat larger than that for the terminal duplex **3:4** (Table 1). Melting temperatures estimated from the temperature dependence of the stilbene 330 nm absorption data are close to those obtained using the nucleotide base 260 nm data for the duplexes (Table 1). Thus the interaction responsible for hyperchromicity in the stilbene absorption band is disrupted only by complete dissociation of the duplex. In the case of the terminal duplex **3:4**, end-fraying of the stilbene must not be extensive below the melting temperature.

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Oligomer Fluorescence. The stilbene-containing oligomers **1**−**4** display fluorescence in the same wavelength region as the model stilbenedicarboxamide **5b** (Figure 3); however, their fluorescence quantum yields are lower than that of **5b** (Table 2). Quenching of the excited singlet and triplet states of chromophores which are covalently attached to nucleosides or oligonucleotides has been extensively investigated.[13,14] In cases where the excited chromophore is a good electron acceptor, quenching has been proposed to occur via an electron transfer mechanism in which a pyrimidine or purine base serves as an electron donor. The relatively high singlet energy (3.4 V) and low reduction potential of the stilbenedicarboxamides (E_{red} = −1.9 V vs SCE for **5a** in dimethyl sulfoxide solution[15]) indicate that the stilbene singlet in oligomers **1**−**4** should function as an electron acceptor. Purines are known to be more readily oxidized than pyrimidines and guanine more readily than adenine.[16,17] The relative quantum yields for fluorescence of the oligomers (**3** > **4** ~ **2** > **1**) are consistent with the presence of guanines as second-nearest neighboring bases on both sides of the stilbene chromophore in **1**, but only one side in **4**. Similar stilbene-containing oligonucleotides in which guanine is the neighboring base are very weakly fluorescent.[11]

The fluorescence decay time of the model stilbenedicarboxamide **5b** is 0.28 ns and its fluorescence quantum yield is 0.11 at 23 °C in aqueous solution (Table 2). These values are larger than those for *trans*-stilbene in organic solvents.[6] The large 4,4′-substituents and specific solvation of the amide and alcohol functional groups in **5b** may result in a larger barrier for torsion about the central double bond that that for *trans*-stilbene, thus increasing the singlet lifetime and fluorescence yield. The observed decrease in lifetime for **5b** with increasing temperature is indicative of an activated nonradiative decay pathway (presumably trans,cis isomerization) which competes with fluorescence. The fluorescence decay of the oligomers **1**−**4** (Table 2) cannot be fit to a single exponential, but can be fit to either a biexponential decay (**2** and **3**) or a triexponential decay (**1** and **4**). A significant short-lived component is observed for oligonucleotides **1** and **4** which possess guanine residues as second-nearest neighbors. All four oligonucleotides display one decay component with a lifetime similar to that of the model stilbene diamide **5b** and one longer lived decay component. These components might arise from conformers which differ in either the proximity of stilbene to different neighboring bases or geometric constraints on stilbene photoisomerization. The weak, long-lived stilbene fluorescence might also arise from weakly-bound exciplexes of the stilbene and neighboring bases. Exciplex fluorescence has been observed by van Houte et al.[13a] for a covalently linked 2-aminofluorene−deoxyguanosine adduct.

Duplex Fluorescence. The fluorescence spectra of the duplexes **1:2** and **3:4** at 0 °C are dominated by broad, long-wavelength emission (Figure 3a,b). The weak shoulder at the high energy end of the fluorescence spectra is attributed to

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scatter and locally excited stilbene fluorescence arising from a slight excess of one oligomer in these solutions. The long-wavelength emission decreases in intensity with increasing temperature (Figure 5) and cannot be detected above the duplex melting temperature, but is regenerated upon cooling. Similarly, the long-wavelength emission is replaced by locally excited fluorescence when the duplex is dissociated by increasing the pH from 7 to 12, and is regenerated when the pH is returned to 7. A duplex formed between oligonucleotide **2** and an analog of **1** containing androstanediol in place of stilbene displays only locally excited stilbene fluorescence. Similarly, stilbene-containing hairpins display only locally excited stilbene fluorescence.[11] Thus the observation of long-wavelength emission requires duplex formation between two stilbene-containing oligonucleotides.

Long-wavelength duplex emission could, in principle, arise from duplex conformations in which the stilbenes are either associated in the ground state or are sufficiently close so that a locally excited stilbene could be quenched by a nearby ground state stilbene in the same duplex. The observation of hyperchromism in the stilbene absorption band supports the former possibility. For the purposes of this discussion, the fluorescent species will be referred to as an excimer, even though this term is normally used for excited dimers whose ground states are dissociative.[18]

Dual exponential excimer fluorescence decay is observed for both **1:2** and **3:4** (Table 3). At low temperatures (0−2 °C) both duplexes display a long-lived component ($\tau \sim 36$ ns) and a shorter-lived component ($\tau \sim 7$ ns). The decay time of the long-lived component of duplex excimer fluorescence decreases with increasing temperature (Table 2), whereas the decay time of the short-lived component is less temperature dependent. The two components make similar contributions to the total fluorescence intensity at several emission wavelengths and thus must have similar emission spectra. The two components plausibly arise from different duplex conformers. Hayashi *et al.*[19] have observed that 1,2-bis(1-anthryl)ethane can form two different intramolecular excimers, which differ in the extent of overlap of the two anthracene rings.

The duplex excimer decay times (Table 3) are significantly longer than the decay times of **5b** or the oligomers **1**−**4** (Table 2). Stilbene monomer singlet lifetimes are normally limited by the rate constant for isomerization[6] and are much shorter than the lifetimes of some stilbene-containing exciplexes.[7b] The long duplex excimer decay times indicate that excimer fluorescence is not effectively quenched by neighboring nucleotide bases. This could result from either restricted mobility within the duplex, which could prevent close approach of the bases and the excimer, or energetically less favorable electron transfer quenching of the excimer by neighboring bases. Atherton and Harriman[20] have suggested that oxidation of duplex base pairs is more difficult than oxidation of the unpaired bases. Reduction of the excimer may also be more difficult than reduction of singlet stilbene because of the lower singlet energy of the excimer.

Photodimerization. Irradiation of the oligomers **1**−**4** results in slow bleaching of the 330 nm absorption band (Figure 7) and the formation of one primary and several secondary products with HPLC retention times similar to those of the reactant oligomers. The primary products are tentatively identified as *cis*-stilbene-containing oligomers on the basis of changes in the appearance of the long-wavelength absorption band, which are similar to those for conversion of *trans*-stilbene to a mixture of cis and trans isomers.

Irradiation of the duplexes **1:2** and **3:4** with 350 nm light results in bleaching of the 330 nm absorption band and disappearance of the excimer fluorescence. As shown in Figure 7, bleaching of the 330 nm absorption band of the duplexes is more rapid and more complete than reaction of the oligomer **2**. Quantum yields for disappearance of the duplexes **1:2** and **3:4** are reported in Table 3. Disappearance of the oligomers and formation of products of higher molecular weight than the oligomers is observed when the photochemical reaction is monitored by IE HPLC (Figure 8). A single product peak is detected for both duplexes by IE HPLC at pH 12; however two peaks of similar retention time are detected for **3:4** by RP HPLC. Under IE conditions (but not RP conditions) the duplex is dissociated.

Hydrolysis of both irradiated duplexes yielded a single isomer of tetrakis(*p*-carboxyphenyl)cyclobutane, identified as **6b** on the basis of identical RP HPLC retention time with a sample obtained from the photodimerization of the stilbene diamide **5a** (eq 1). Photodimerization is assumed to occur via a singlet excimer, as proposed for the photodimerization of *trans*-stilbene in organic solvents.[7] The observation of oxygen quenching of both excimer fluorescence and photodimerization provides support for this assumption. We tentatively assign dimer formation to the shorter lived of the two fluorescent excimers on the basis of the absence of temperature dependence of either the lifetime of this excimer or the quantum yield for photodimerization (Table 3). The observation of two product peaks from irradiated solutions of **3:4** by RP HPLC but only one photodimer presents something of a puzzle. Different geometries for attachment of the cyclobutane to the duplex might give rise to two diastereotopic duplexes. Disruption of the duplex structure under IE HPLC conditions could eliminate this structural difference and alkaline hydrolysis yields a single cyclobutane.

Stilbene Excimers. Stilbene excimer fluorescence has not been observed to accompany self-quenching of *trans*-stilbene in homogeneous solution.[7] Since the limiting quantum yield for photodimerization of *trans*-stilbene in benzene solution is 1.0,[7a] the absence of excimer fluorescence may be a consequence of rapid excimer dimerization, as is the case for the anthracene excimer at room temperature in solution.[19] Peters et al.[7c] have observed an intermediate in the photodimerization of stilbene by means of transient absorption spectroscopy. This intermediate, which might be either the excimer or a biradicaloid, decays with a rate of 2.4×10^9 s^{-1}. Excimer fluorescence has been reported for polymers with high concentrations of pendant stilbene chromophores.[8] In the case of a stilbene-containing polypeptide, the stilbene fluorescence changed from that of the monomer to that of the excimer as water was added to a trimethyl phosphate solution. The effect of added water was attributed to hydrophobic association of the stilbene chromophores.

Evidence for ground state interaction of the two stilbene chromophores in the duplexes **1:2** and **3:4** is provided by the broadening and hypochromicity of the stilbene absorption band observed upon mixing the oligomers. Broadened stilbene absorption has been observed by Whitten and co-workers[9] for aqueous solutions of the bis-stilbene phospholipid **8** in the presence of excess saturated phospholipid or in the presence of high concentrations of γ-cyclodextrin. In the absence of excess saturated phospholipid or cyclodextrin, **8** forms aggregates with blue-shifted absorption characteristic of an "H" aggregate or

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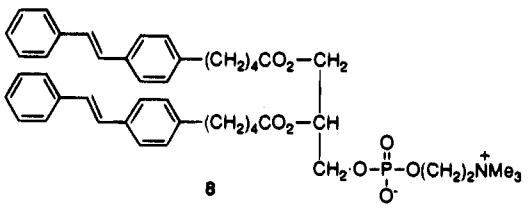

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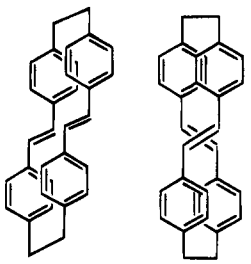

9a **9b**

"card pack" array. A broadened absorption spectrum has also been recently reported by Angler et al.[10] for the stilbeneophane **9**.

A mixture of locally excited and excimer fluorescence has been observed for the phospholipid **8**[9] and the stilbeneophane **9**.[10] Angler et al.[10] have proposed that the two conformers of **9** having parallel (**9a**) and crossed (**9b**) double bonds are both fluorescent at low temperatures, but only one forms a fluorescent excimer. The lifetime assigned to the excimer of **8** is 4.4 ns[9] and the fluorescence decay assigned to the excimer of stilbeneophane **9** has a temperature-dependent lifetime, decreasing from 39 ns at 153 K to 1.4 ns at 298 K in dichloromethane solvent.[10] The stilbenophane **9** is reported to undergo efficient trans,cis photoisomerization at room temperature. Photodimerization has not been reported for either **8** or **9**.

The excited state behavior of the duplexes **1:2** and **3:4** differs in several significant respects from that of **8** or **9**. The red-shift for excimer vs monomer fluorescence is much larger for the duplexes than for **8** or **9**. Formation of a more stable excimer is consistent with the larger excimer:monomer fluorescence ratio and the longer excimer lifetimes for the duplexes. In addition, the excited duplexes undergo moderately efficient photodimerization but little or no photoisomerization, whereas **9** undergoes isomerization but not dimerization. These differences in behavior may reflect differences in both structure and environment. The structure of **9a** has been determined by X-ray crystallography. The two stilbenes are bowed out by ring strain and the ethylene−ethylene distance is 4.4 Å,[21a] slightly larger than the optimum distance for photodimerization.[21b] The structure of the ground state dimers formed by the duplexes **1:2** and **3:4** is not known; however, amide−amide hydrogen bonding as well as hydrophobic association between neighboring stilbenes may contribute to their ground state stabilization. Investigation of the solid state and solution structure and photochemistry of **5a** is currently in progress in our laboratories.[15]

It is tempting to assign the shorter-lived and longer-lived excimer fluorescence decay to parallel and crossed stilbene geometries similar to those of **9a** and **9b**, respectively. Moderately efficient photodimerization of the excimer with parallel geometry would account for both the shorter excimer lifetime and the observed stereochemistry of photodimerization. Photodimerization of the excimer with crossed geometry would require that the carboxamide linkers on opposite oligomers move apart to accommodate the *all-trans* tetraarylcyclobutane structure, a motion resisted by the constraints imposed by the duplex geometry.

Duplex Formation and Cross-Linking. The changes in the absorption and fluorescence spectra which occur upon hybridization of the complementary stilbene-containing oligonucleotides are consistent with a simple two-state model for duplex formation. Below the duplex melting temperatures the stilbene chromophores are held in close proximity and display strong excimer emission whereas only locally excited stilbene fluorescence is observed above the duplex melting temperatures (Figure 5). The absence of appreciable locally excited fluorescence below the melting temperature, like the hypochromicity of the stilbene 330 nm absorption band (Figures 1 and 2), supports the conclusion that looped-out conformations of the mid-strand duplex **1:2** and end-frayed conformations of the terminal duplex **3:4** are not present in significant populations. As a consequence, the temperature dependence of the locally excited fluorescence intensity (Figure 6) can provide information about exciplex dissociation. The melting temperature estimated from the inflection point of Figure 6 for 5×10^{-6} M duplex **3:4** is 45 °C, essentially the same as that obtained from the absorption data (Table 1). A lower melting temperature is observed for the 5×10^{-7} M duplex, reflecting the normal concentration dependence for association.

Covalently attached fluorescent probes have been widely used to investigate duplex formation and structure.[22-24] Fluorophore-labeled oligomers can display significant increases (or decreases) in fluorescent intensity upon hybridization with an unlabeled complementary oligomers.[22] Doubly labeled oligomers with a fluorophore and quencher on opposite ends can display increased resonance fluorescence energy transfer (FRET) upon duplex formation.[23] FRET has also been used to investigate duplex formation and dissociation of oligomers in which the 5′ end of one oligomer and the 3′ end of its complement are both labeled with fluorophores.[24] To our knowledge, there are no previous examples of excimer fluorescence resulting from hybridization of complementary oligonucleotides conjugates.[25] While the utility of excimer fluorescence as a probe of duplex formation and structure remains to be investigated, it offers potential advantages over single labels and the use of FRET. For example, excimer emission is observed only for the duplex thus it is not necessary to correct the observed signals for background emission from uncomplexed oligomers. In addition, the short stilbene monomer lifetime renders collisional quenching improbable.

Stilbene photodimerization results in a marked increase in the duplex melting temperature (Table 1). The photodimers of duplexes **1:2** and **3:4** can be viewed as double and single hairpins, respectively. The higher melting temperature for the photodimer of **3:4** vs **1:2** is consistent with its longer duplex sequence.[11] The increase in melting temperature upon cross-linking of duplex **1:2** is similar to that for covalently capping an oligonucleotide duplex with a stilbenedicarboxamide bridge[11]

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or for cross-linking of a decanucleotide with a disulfide bond.[26] Photochemical cross-linking of double stranded regions of DNA and RNA using intercalating agents such as the psoralens has been extensively used to probe the secondary structure of large RNA molecules.[27] Cross-linking of psoralen-labeled oligonucleotides complexed with single-stranded DNA has also been reported.[28] Covalently attached photosensitizers have also been used to effect cross-linking of duplex DNA.[29] Stilbene photodimerization may prove useful in applications such as cross-linking relatively unstable duplexes, cross-linking single-stranded hairpins containing two stilbenes, and ligating two stilbene-labeled oligomers which bind to single stranded DNA at adjacent binding sites. These and other applications are being investigated in our laboratories.

Experimental Section

General Methods. The synthesis and purification of stilbene-containing oligonucleotides is described elsewhere.[11] Samples were analyzed for purity and for product formation by reverse phaes HPLC and ion exchange HPLC. HPLC analyses employed 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 (pH 7.0) with a flow rate of 1.0 mL/min (reverse phase) or a Dionex NucleoPac PA-100 column (4.0 × 250 mm) and a 2%/min gradient of 1.5 M NaCl in 10 mM NaOH (pH 12.0) with a flow rate of 1.0 mL/min (ion exchange). Samples were stored in the dark and protected from exposure to stray light during analysis.

Ultraviolet absorption spectra were recorded using a Hewlett-Packard 8452 diode-array spectrophotometer or a Perkin Elmer Lambda 2 spectrophotometer equipped with a temperature programmer for automatically increasing the temperature at the rate of 0.8 °C/min. The latter instrument was used for melting temperature measurements.

Fluorescence spectra were obtained on a Spex Fluoromax or a Perkin Elmer LS 50B spectrofluorimeter equipped with a RM6 Laude circulating bath for temperature control. Fluorescence decay times were measured with two different single-photon counting apparatuses with different excitation sources, one with a gated arc lamp (PTI-LS1, time resolution ca. 0.2 ns) and the other with a mode-locked dye laser (time resolution ca. 50 ps). The method of analysis of the fluorescence decay curves has previously been described.[30] Fluorescence spectra were recorded on oligonucleotide samples dissolved in an aqueous solution containing phosphate buffer (10 mM, pH 7) and 1 M NaCl. Optical densities were adjusted to ca. 0.1 A_{260} units. Fluorescence quantum yields of the oligonucleotides and duplexes were determined by matching optical densities at 330 nm with phenanthrene in cyclohexane solution ($\Phi_f = 0.13$[31]) and measuring the ratio of the total emissive output, corrected for differences in solvent refractive index. Solutions in cuvettes equipped with serum caps were deoxygenated by slowly purging with purified nitrogen.

Photobleaching experiments were performed using a Rayonet reactor with 350 nm lamps. Quantum yields for disappearance of duplex were determined for solutions with an optical density of 0.2 at 330 nm using an optical bench apparatus equipped with a 200W xenon−mercury high-pressure lamp, 0.25 M monochrometer, and a thermostated, stirred cell holder. Photon fluxes were measured using Aberchrome chemical actinometry[32] to determine the lamp output as well as the amount of light transmitted. The experimentally determined amount of light transmitted during irradiation agreed well with the calculated value determined by measuring the optical density at the midpoint of the irradiation. The extent of reaction was monitored by both UV and HPLC. There was excellent agreement between the extent of reaction determined by these methods.

Isolation of Photodimers. A solution of 4,4′-*N*,*N*-dimethylstilbenedicarboxamide (**5a**, 0.02 M in 5 mL of dimethyl sulfoxide) was irradiated at room temperature for 14 h with the Pyrex-filtered output of a 450W Hanovia medium-pressure mercury lamp. The solution was added to 10 mL of 3 M aqueous NaOH and refluxed overnight. The solution was cooled and acidified. The resulting precipitate was digested with methanol to separate the soluble dimers from the insoluble stilbenedicarboxylic acid. The methanol solution was evaporated to dryness and dissolved in DMSO-d_6. The ^{1}H NMR spectrum was assigned, by analogy to the reported spectra of **6c** and **7c**,[7d] to a mixture of **6b** [δ 12.86 (bs, 4H), 7.71 (d, J = 8.4 Hz, 8H), 7.34 (d, J = 8.4 Hz, 8H), 4.72 (s, 4H)] and **7b** [δ 12.86 (bs, 4H), 7.89 (d, J = 8.3 Hz, 8H), 7.53 (d, J = 8.3 Hz, 8H), 3.85 (s, 4H)]. Analysis of this mixture dissolved in 50 mM phosphate buffer (pH 9.2) by RP HPLC provided retention times of 14.3 and 15.3 min for **6b** and **7b**, respectively. The ratio of isomers obtained by RP HPLC was similar to that obtained by NMR integration for two solutions with **6b**:**7b** ratios of 1:1.3 and 1:0.86, respectively. As a test of the recovery procedure and HPLC analysis, a 1 mL aliquot of this solution with $A_{250} = 0.72$ was acidified and centrifuged as described below. A solution of the precipitated material showed some loss of material ($A_{250} = 0.56$) but no change in the ratio of isomers as determined by HPLC.

A solution of oligonucleotides **1** and **2** ($A_{260} = 2.0$) dissolved in 1 mL of 0.1 NaCl and 10 mM tris·HCl (pH 7.0) in a quartz cell was irradiated at 350 nm in a Rayonet reactor. Monitoring the long-wavelength absorption band showed that most of the stilbene chromophores had reacted within 2 min. The product was isolated by RP HPLC (retention time, 19.2 min) and the collected fractions lyophilized, dissolved in 0.5 mL of 3 M aqueous NaOH, and heated at 70 °C for 12 h. Analysis of the resulting solution by RP HPLC revealed the formation of a complex mixture of UV-absorbing species. The solution was neutralized with 3 M HCl, acidified to pH 2 by adding 10 μL of 1 M HCl, cooled to 0 °C for 30 min, and centrifuged. The supernatant was removed and the residue redissolved in 1 mL of 50 mM phosphate buffer (pH 9.2). Analysis of this sample ($\lambda_{max} = 244$ nm, $A_{244} = 0.5$) by RP HPLC revealed the presence of one major species (retention time, 15.2 min). Co-injection with the mixture of **6b** and **7b** showed this product corresponds to **6b**.

A slightly longer irradiation period was required for bleaching of the long-wavelength absorption band of a solution of **3** and **4**. Analysis of the irradiated solution by RP HPLC indicated the formation of two products in comparable yield (retention times, 20.1 and 20.4 min), whereas a single product peak was detected by IE HPLC at pH 12. Alkaline hydrolysis and workup as described above provided a sample containing one major species with the same RP HPLC retention time as that obtained upon hydrolysis of the photoproduct of the mixture of oligonucleotides **1** and **2**.

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