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CHEMICAL AND PHOTOCHEMICAL LIGATION OF OLIGONUCLEOTIDE BLOCKS

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Abstract: Several efficient means for joining oligonucleotides in dilute solution by non-natural internucleotide bridges are discussed. It is also shown that an oligonucleotide containing a -OP(O)(O⁻)S- link can function as an effective template in PCR amplification and that oligonucleotide probes containing stilbenedicarboxamide groups can serve in monitoring the presence of mismatched bases in an oligonucleotide target.

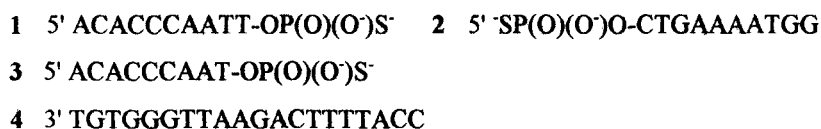
Template induced chemical ligation can play a role in synthetic methodology as a complement for enzymatic ligation. Of several chemical methods described for generating phosphodiester links between oligonucleotides,¹ the most successful involves activation of a terminal phosphoryl groups with cyanogen bromide.^{1c} Rapid and efficient ligation has been reported for a number, but not all, cases with this reagent.

In principle, ligation can be useful in covalently locking in place novel supramolecular structures organized by hybridization of oligonucleotide segments as well as in synthesizing natural polynucleotides. For this purpose, neither the enzymatic nor the cyanogen bromide procedures may be suitable. Both require a close fit of the ligating strands on the template, a condition that would not obtain in many cases, and the yields are variable. To provide more versatility in synthetic methodology for joining oligonucleotides, both for biochemical and chemical applications, we have investigated several chemical and photochemical methods that afford non-natural internucleotide linkages.² In the present paper we (1) summarize the results of our previous work in this area, (2) report data on the effects of the unnatural bridges on PCR amplification, and (3) describe a photochemical ligation system exhibiting fluorescence and coupling properties sensitive to point mutations in the target strand.

Chemical Ligation. Three systems are indicated in TABLE 1, where R and R' represent oligonucleotides. The functional group chemistry has precedents. We asked whether

such chemistry might be exploited in linking oligonucleotides organized by hybridization in dilute solution and how the unnatural bridges affect properties of the products.

We found that the rates of coupling are indeed greatly enhanced when the reactants are aligned by hybridization on a complementary oligonucleotide; that the yields for the template directed reactions are high (>90 %); and that the systems do not require a perfect fit at the ligation site. For example, oxidation of **1** + **2** and of **3** + **2** in the presence of template **4** (each oligonucleotide at 2 μ M) at 0 °C for 5 minutes afforded >90% yield of coupling products **6** and **7**, respectively,³ even though template **4** has an extra base opposite the ligation site for **3** + **2**. Introduction of the -S-S- (*a*) and amidate (*b*) bridges lead to some destabilization of duplexes formed with complementary natural oligonucleotides. For products obtained by ligating two ten-mer blocks the T_m values dropped from 60 °C for the natural duplex to 56 °C for a duplex containing the amidate bridge and 55 °C for one containing the -S-S- bridge. No effect on T_m values was observed when oligonucleotides were linked by the thiophosphoryl bridge (*c*).



Each of the ligations indicated in TABLE 1 has features that make it unique. Both the phosphorothioate oxidative coupling and the displacement on the bromoacetamido group proceed very rapidly in the duplex, even at 0 °C, whereas the tosylate displacement requires >1 hour at room temperature. The phosphorothioate coupling is reversible in the sense that the S-S product undergoes exchange with oligonucleotides bearing terminal phosphorothioate groups, whereas the other couplings are irreversible. A distinguishing feature of the systems affording *b* and *c* bridges is that the reactions are autoligations, that is, they take place spontaneously in the presence of the template without need for addition of another reagent. This feature opens the possibility of carrying out chemical ligation in biological systems. The tosyl oligonucleotides, although less reactive than the bromoacetamido derivatives, are easier to prepare and manipulate. They can be prepared on solid supports using amidite reagents and recovered by treatment with ammonium hydroxide, whereas the bromoacetyl group has to be introduced after isolation of the oligonucleotide. Also, as a consequence of their relative stability the tosyl derivatives are well suited for cyclization reactions, where both the reactive nucleophile and the displaceable group are present in the same molecule. Examples of

TABLE 1. Chemical Ligation in Presence of Complementary Templates

Oligonucleotides	Condensing Reagent	Product (bridge)	Reference
$\text{ROP}(\text{O})(\text{O}')\text{S}^- + \text{'SP}(\text{O})(\text{O}')\text{OR}'$	I_3^- or $\text{Fe}(\text{CN})_6^{3-}$	$\text{ROP}(\text{O})(\text{O}')\text{S-SP}(\text{O})(\text{O}')\text{OR}'$ (<i>a</i>)	3
$\text{ROP}(\text{O})(\text{O}')\text{S}^- + \text{BrCH}_2\text{C}(\text{O})\text{NHR}'$	none	$\text{ROP}(\text{O})(\text{O}')\text{SCH}_2\text{C}(\text{O})\text{NHR}'$ (<i>b</i>)	4
$\text{ROP}(\text{O})(\text{O}')\text{S}^- + \text{Tosyl-OR}'$	none	$\text{ROP}(\text{O})(\text{O}')\text{SR}'$ (<i>c</i>)	5

application of this chemistry include synthesis of a 24-nucleotide dumbbell, closure of a ring containing two stilbenedicarboxamide groups and unmatched bases in the sequence,^{5a} and cyclization of an oligonucleotide conjugate containing only four nucleotides.^{5b}

PCR with Bridged Oligonucleotides. In some applications it would be helpful to employ the ligated oligonucleotides as templates for synthesis of oligonucleotides by PCR amplification. The products from the ligations listed in Table 1 all contain unnatural bridging elements. To see whether the Taq enzyme employed in PCR would read through these modifications we prepared modified oligonucleotide templates **5a,b,c** and **6a,b,c**, where X represents linkers $-\text{OP}(\text{O})(\text{O}')\text{S-SP}(\text{O})(\text{O}')\text{O}-$, $-\text{OP}(\text{O})(\text{O}')\text{SCH}_2\text{C}(\text{O})\text{NH}-$, and $-\text{OP}(\text{O})(\text{O}')\text{S-}$ for **a**, **b**, and **c**, respectively. These oligomers were obtained by chemical ligation on 16-mer templates that overlapped both modified strands to be joined. The enzymatic amplifications were carried through 30 cycles using pairs of templates containing the same modification (1.3 nM each) and primers **7** + **8** (1 μM each), and products were analyzed by ion exchange HPLC at pH 12. For comparison, natural templates **5d** and **6d**, where $\text{X} = -\text{OP}(\text{O})(\text{O}')\text{O}-$, were also examined. The T_m values (0.1 M NaCl, pH 7.0) for duplexes formed from **5n** + **6n** containing linkers **a**, **b**, **c**, and **d**, respectively, were 70 °C, 71 °C, 73 °C, and 73 °C.

Templates **5a-d**: 5' AGTTAAATAGCTTGCAAAA-X-TACGTGGCCTTATGGTTACAG

Templates **6a-d**: 3' TCAATTTATCGAACGTTTT-X-ATGCACCGGAATACCAATGTC

Primer **7**: 5' AGTTAAATAGCTTGC Primer **8**: 5' CTGTAACCATAAGGC

The HPLC profiles for the products are presented in FIG. 1. The primers eluted at 19 and 21 minutes and the oligonucleotide products (40-mers) eluted at 25 and 26 minutes. In

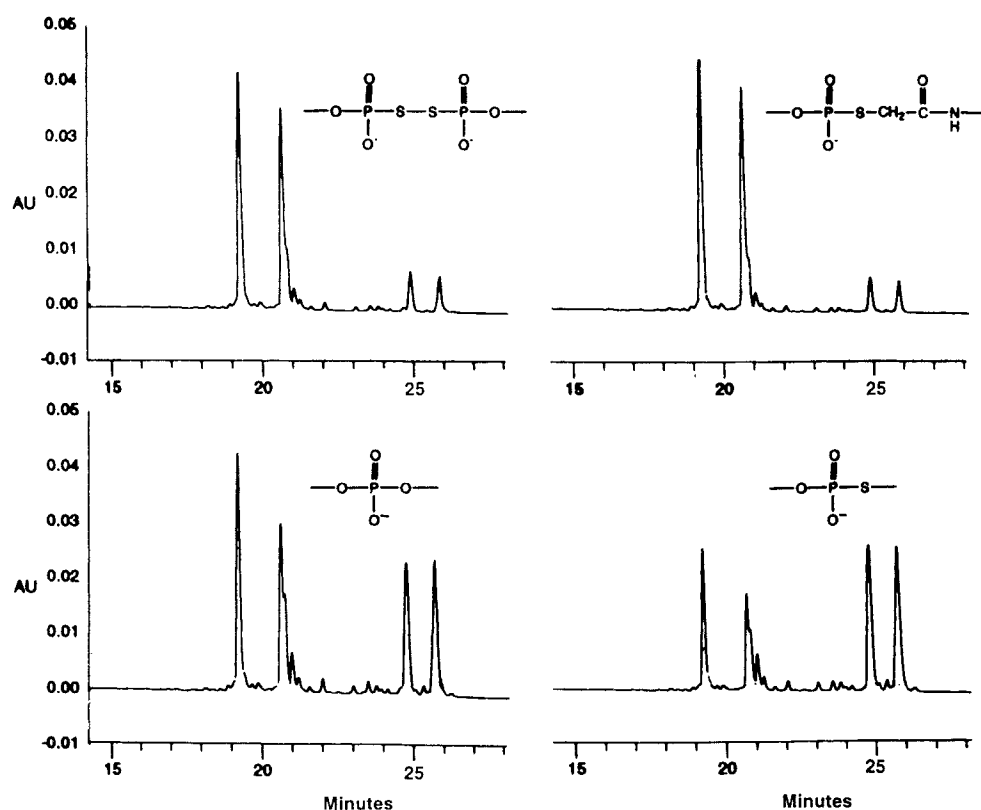
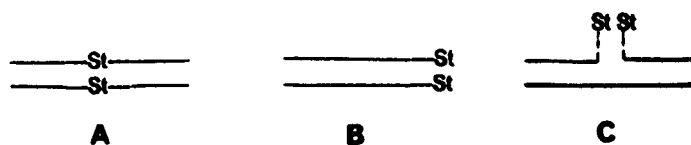


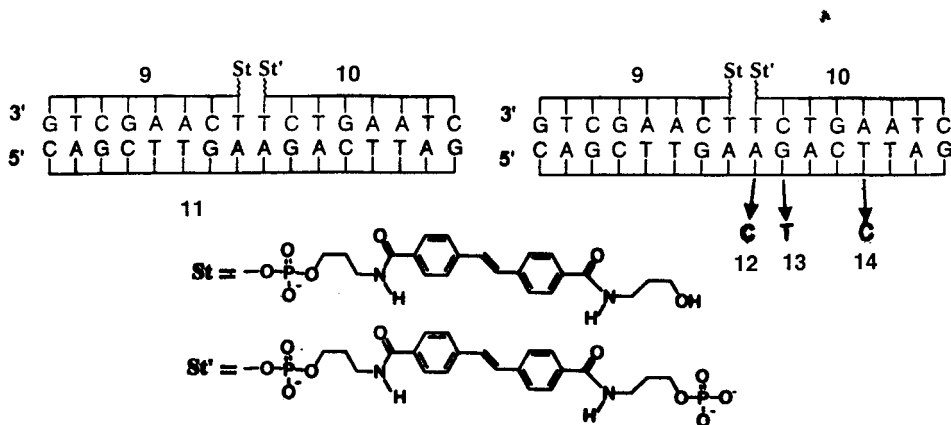
FIG 1. Ion Exchange HPLC profiles (pH 12) for products of PCR using templates with connecting internucleotide links X = $-\text{OP}(\text{O})(\text{O}^-)\text{S}-\text{SP}(\text{O})(\text{O}^-)\text{O}-$, $\text{ROP}(\text{O})(\text{O}^-)\text{SCH}_2\text{C}(\text{O})\text{NHR}$, $-\text{OP}(\text{O})(\text{O}^-)\text{O}-$, and $-\text{OP}(\text{O})(\text{O}^-)\text{S}-$.

control experiments it was demonstrated that the signals from the templates were too weak to observe at the nM concentrations employed and that PCR carried out with primers in absence of template exhibited peaks for the primers but no later eluting material. The data in FIG. 1 show that amplification of the 40-mer sequences was achieved in each case, but the extent of synthesis using templates containing the disulfide and the amidate bridges was low. The noteworthy finding is that the $-\text{OP}(\text{O})(\text{O}^-)\text{S}-$ bridge is as effective as $-\text{OP}(\text{O})(\text{O}^-)\text{O}-$ in these systems. Less efficient synthesis in the other cases is not surprising since considerable distortion in duplex geometry at the X site would be expected for these extended bridges.

Photoligation. Stilbenedicarboxamide groups (abbreviated St) joined through linkers to oligonucleotides and aligned by hybridization, as in constructs **A** and **B**, exhibit two striking



SCHEME 1: Schematic representation of duplexes formed from oligonucleotides linked to stilbenedicarboxamide groups



SCHEME 2. Compounds used in investigating properties of stilbenedicarboxamide groups aligned as in construct C.

properties - a shift in the fluorescence maximum from that for the stilbenedicarboxamide group at ~ 390 nm to ~ 450 nm, attributable to excimer fluorescence, and efficient photodimerization of the adjacent stilbene moieties ($\Phi \sim 0.2$).⁶ In view of these facts, it appeared that stilbenedicarboxamide oligonucleotide conjugates hybridized on an oligonucleotide target, as in construct C, might both exhibit excimer fluorescence indicative of hybridization to a non-conjugated target oligonucleotide and undergo a photoinduced ligation. This concept was tested with complexes formed by conjugates **9**, **10**, and target **11** (SCHEME 2).

As shown in FIG. 2, oligomers **9** and **10** in absence of template and the complexes formed from **9** + **11** and **10** + **11** exhibited fluorescence characteristic of the stilbenedicarboxamide group; however, an additional fluorescence band ($\lambda_{\max} \sim 450$ nm) appeared in the spectrum of the ternary complex formed from **9** + **10** + **11**. This longer wavelength band corresponds to the excimer fluorescence observed for constructs **A** and **B**.⁶ The excimer

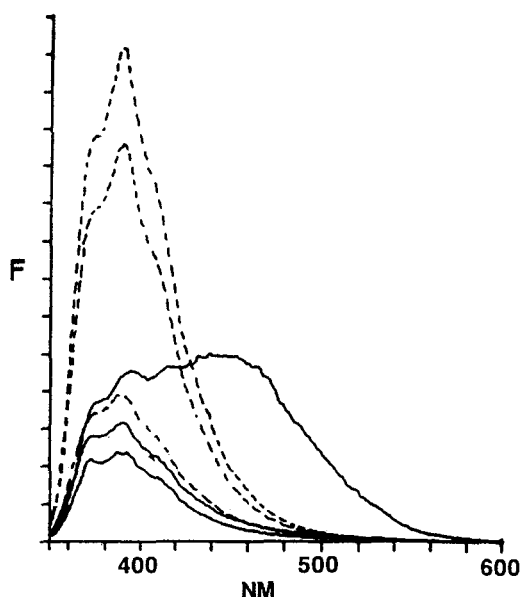


FIG. 2. Fluorescence spectra at 0°C, 1.0 M NaCl: top to bottom at 400 nm, **9+10**, **10**, **9+10+11**, **9**, **10+11**, **9+11**

emission is distinctive, although relatively less pronounced than in the case of construct **A**,⁶ suggesting more disorder in alignment of the stilbenedicarboxamide groups in this complex than in **A**. A difference in alignment of the chromophores is also indicated by the differences in the induced CD spectra in the 300–380 nm region for the different systems (FIG. 3, see ref. 6b for sequences for **A** and **B**). No induced CD bands were exhibited by the individual oligonucleotide conjugates.

The photochemistry of the ternary complex paralleled that for stilbenedicarboxamide groups aligned as in **A** and **B**. Irradiation of the complex (**9 + 10 + 11**) at $\lambda > 300$ nm led to a rapid decrease in absorbance at 335 nm (FIG. 4), an increase in T_m for dissociation of the complex from 44 °C to 55 °C (1.0 M NaCl, pH 7.0), a marked decrease in intensity of the peaks in the IE HPLC profile for conjugated strands **9** and **10** relative to template strand **11**, and appearance of a large new peak in the IE HPLC profile eluting later than the template strand (FIG. 4). These facts, in conjunction with the studies on related systems **A** and **B**,⁶ provide strong evidence for a template directed photoligation of **9** and **10** that proceeds by cross-linking

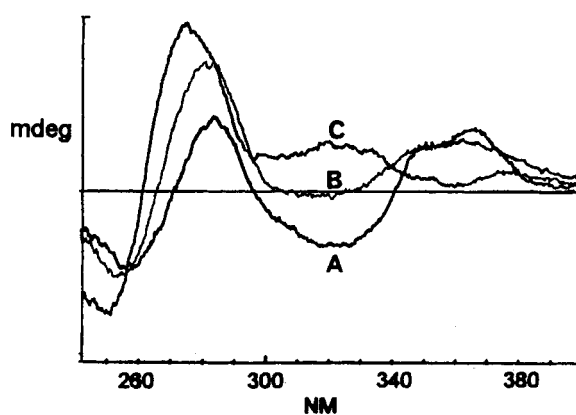


FIG.3. CD spectra for A, B, and C.

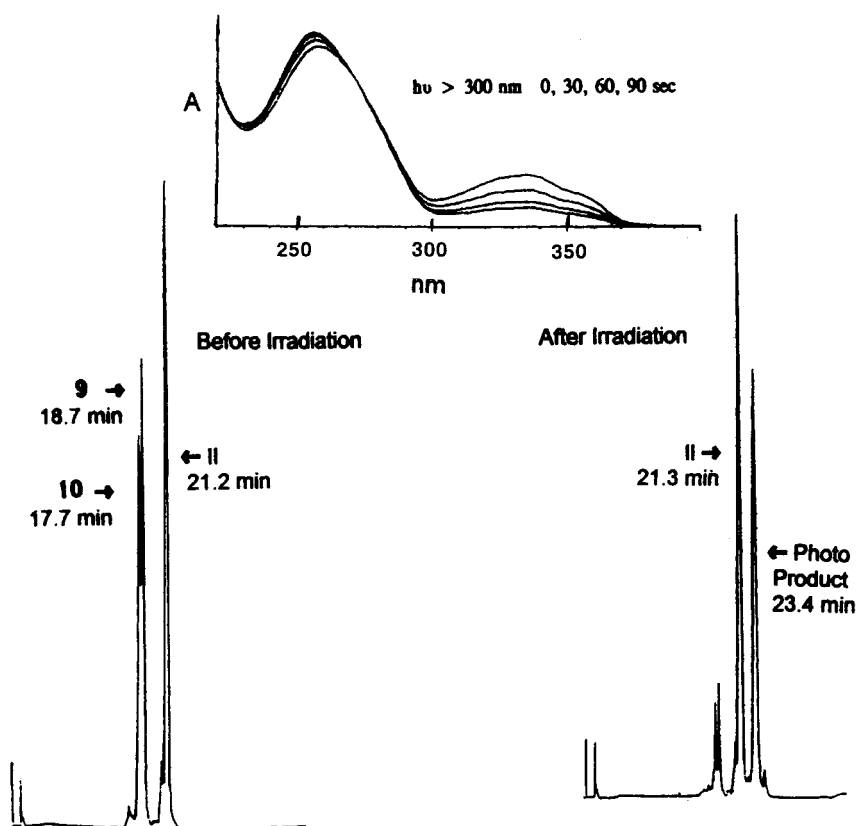


FIG. 4. UV spectra (inset) and IE HPLC profiles (pH 12) for reactants and products of photoligation of 9+10+11.

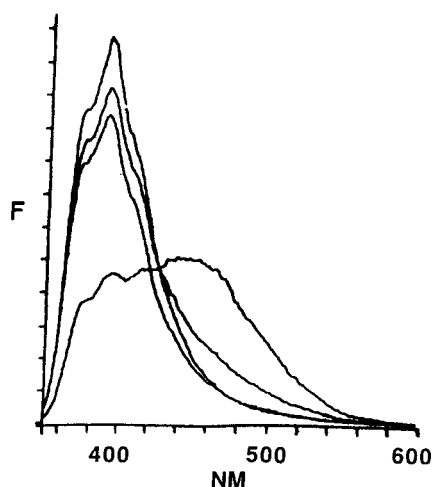


FIG. 5. Fluorescence spectra, 0°C, 1.0 M NaCl, of complexes containing templates **13**, **14**, **12**, and **11**, reading from top to bottom at 400 nm.

the stilbene groups.⁷ Additional control experiments support this conclusion. Thus, no slow eluting product was observed when **9** + **10** was irradiated in absence of the template or when **9** + **11** was irradiated in absence of **10**. In these cases only a low conversion to photoproducts eluting about half a minute before the conjugates (**9** or **10**) on IE HPLC was observed. These products are probably *cis* isomers of **9** and **10** formed by photoisomerization of the stilbenedicarboxamide group.⁸

The effects of single base mismatches in the template strand was investigated by study of the complexes formed from conjugates **9** and **10** hybridized to targets containing a single mismatched base (SCHEME 2). In target **12** an adenine residue that pairs with a thymine near the junction site of the probes in the fully matched ternary complex has been replaced by cytosine; in target **13** a nearby guanine has been replaced by thymine; and target in target **14** a more distant thymine has been replaced by cytosine. These structural changes led to significant and easily observable changes in the fluorescence and photochemical properties of the system. In each case the fluorescence corresponded predominantly to that for the isolated chromophore (FIG. 5) and photoligation was minimal (FIG. 6). The modifications near the ligation site (targets **12** and **13**) had the greatest effect on photoligation.

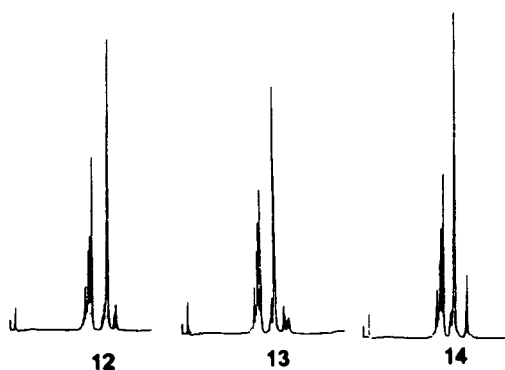


FIG. 6. IE HPLC of irradiation products of complexes containing templates 12, 13, and 14.

Summary. A photochemical and three chemical methods for efficiently linking oligonucleotides organized by hybridization in aqueous solution are described. The photochemical method, based on alignment of two stilbenedicarboxamide groups, is sensitive to base mismatches in the target oligonucleotide. Point mutations in the target can be detected both by absence of excimer fluorescence and by inefficient ligation. The chemical procedures depend on hybridization but do not require a perfect fit to the template. Linkage by displacement of a tosyl group by a terminal phosphorothioate group is particularly attractive for generating cyclic oligonucleotides and covalently locking in place novel self-assembled structures. The internucleotide linkage that is formed closely resembles the natural phosphodiester linkage. It does not interfere with PCR amplification and does not destabilize hybridization of oligonucleotides containing this bridge.

Experimental Section. Oligonucleotides, stilbenedicarboxamide conjugates,⁸ and tosyl and phosphorothioate derivatives⁵ were prepared and characterized by the methods employed for related compounds. Melting curves, HPLC, spectral determinations, and photochemical experiments were carried out as described.^{6,8} Photoligations were carried out in 1.0 M aqueous NaCl at pH 7.0 (10 mM phosphate) using two 350 Å Rayonet lamps. PCR amplifications were carried out using a Perkin Elmer Ampli Taq PCR kit. In each case a solution (100 μ L) containing templates (1.3 nM each), primers (1 μ M each), the four d-NTP's (200 μ M each) and enzyme (5 units) was cycled 30 times at 94 °C (denaturation), 37 °C (annealing), and 72 °C (polymerization) with 1 minute duration at each stage.

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