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Template controlled coupling and recombination of oligonucleotide blocks containing thiophosphoryl groups

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ABSTRACT

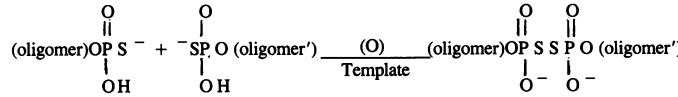
Oxidation of a pair of 3′- and 5′-thiophosphoryloligonucleotides in the presence of a complementary oligonucleotide template is shown to provide an effective means for selectively linking oligonucleotide blocks. Coupling proceeds rapidly and efficiently under mild conditions in dilute aqueous solutions (μM range for oligomers, 2–15 min at 0–4°C with $K_3Fe(CN)_6$ or KI_3 as oxidant). This chemistry was demonstrated by polymerization of a thymidylate decamer derivative (sTTTTTTTTTTs) in the presence of poly(dA) and by coupling oligomers possessing terminal thiophosphoryl groups (ACACCCAATTs + sCTGAAAATGG and ACACCCAATs + sCTGAAAATGG) in the presence of a template (CCATTTTCAGAATTGGGTGT). Efficient linking of 5′ to 3′ phosphoryl groups can be achieved under conditions where virtually no coupling takes place in absence of a template. A novel feature of the chemistry is that catalyzed recombination of oligomers containing internal -OP(O)(O⁻)SSP(O)(O⁻)O- linkages can be directed by hydrogen bonding to a complementary oligonucleotide. Convenient procedures are reported for solid phase synthesis of the requisite oligonucleotide 3′- and 5′-phosphorothioates.

INTRODUCTION

The first example of a template directed ligation of oligonucleotides was reported in 1966 by Naylor and Gilham. (1) Using a chemical activating agent (a water soluble carbodiimide), they obtained a 5% yield of a thymidine dodecamer by coupling thymidine hexanucleotides on a poly(A) template. Soon after, following isolation and characterization of *E.coli* (2–5) and T4 (6,7) DNA ligase, a practical enzymatic procedure was developed for ligating oligonucleotides. (8) The enzymatic method has since played a pivotal role in processing oligonucleotides for many applications in molecular biology. By contrast, approaches for template mediated chemical ligation of oligonucleotides have evolved slowly. The most effective coupling reagents have proven to be N-cyanoimidazole, (9,10) cyanogen bromide, (11) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. (12) Although a rapid reaction involving cyanogen bromide has been reported, (11) generally hours to days are required to achieve respectable yields in these systems. A useful feature of chemical coupling is that modified internucleoside links, such as phosphoramidate, (12,13) pyrophosphate, (12) and methyl pyrophosphate, (14) as well as the natural phosphodiester link can be generated. Practical progress in the field is indicated by the fact that chemical ligation has recently been employed in synthesizing defined oligomers targeted for physical and biochemical studies. (14–16).

Eckstein reported in 1966 that oxidation with $K_3Fe(CN)_6$ converts thymidine 5′-phosphorothioate to a dimer derivative possessing a -OP(O)(O⁻)SSP(O)(O⁻)O- bridge. (17) Recently Wu and Orgel used this chemistry in the cyclization and polymerization of deoxyadenosine 3′,5′-bisphosphorothioate. (18) We report here that, when directed by a template, oxidative coupling of phosphorothioate derivatives provides an excellent means for selectively linking oligonucleotide blocks differing in sequence. The net reaction is indicated in Chart 1. Distinguishing features of this approach are: template directed oxidative couplings take place rapidly and selectively at low temperature even in very dilute aqueous solutions of unprotected oligomers; the products are stable over a wide pH range, yet they can be cleaved rapidly and quantitatively at a specific position (the S-S bond) by mild reducing agents; and, in contrast to the electrophilic condensing agents employed in conventional chemical ligation, the 'activating agents' are not destroyed by water.

Chart 1



MATERIALS AND METHODS

General methods

HPLC was carried out on a Dionex chromatograph. A Hypersil ODS column (4.6×200 mm, 5μ particle size) with a 1%/min gradient of CH_3CN in 0.03 M $Et_3NH^+OAc^-$ buffer (TEAA), pH 7.0, was used for RP HPLC. For ion exchange chromatography a Dionex Omni Pak NA 100 column (4×250 mm) was used with gradient *A* (2.0%/min of 1.5 M NaCl in 0.03 M TEAA, pH 7.0), gradient *B* (2%/min of 1.5 NaCl in 0.01

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M NaOH in 0.01, pH 12), or gradient C (2%/min of 1.0 M NaCl in 0.01M NaOH, pH 12). Thin layer chromatography (TLC) was carried out on DC-Fertigplatten SIL G-25 UV_{254} plates (Macherey–Nagel) with iPrOH/NH_4OH/H_2O 7/1/2 v/v/v as eluent. NMR spectra were run on a Varian XL400 instrument with CD_3CN or D_2O as solvent and 85% H_3PO_4 as an external standard.

A 'standard buffer', pH 7.05, 15 mM sodium phosphate with NaCl to make $[Na^+]$ = 100 mM, was used for all thermal dissociation experiments. For runs carried out after oxidation with $K_3Fe(CN)_6$ or KI_3, the products were desalted on a NAP-5 column, concentrated to dryness, and taken up in 1 ml of buffer for the measurement. Most of the data were collected on a Perkin Elmer Lambda 2 spectrophotometer equipped with a temperature programmer and Dec 316 computer for automatic ramping of temperature and data processing. Absorbance values at 260 nm were obtained at 2 min intervals with a temperature increase of 0.81°C/min. For data in Figure 1 a Perkin Elmer 570 instrument was used and data points were collected as the temperature was increased incrementally 5°C at 5 min intervals.

Oligonucleotide 3′-phosphorothioates

The first steps were carried out on 100 mg of LCAA CPG (500 Å, Sigma) in a MilliGen-Biosearch column connected to a syringe for drawing in and expelling reagents. The loaded support was treated with 250 μl of 0.1 M DMT-$OCH_2CH_2SO_2CH_2CH_2OP$-$(OCH_2CH_2CN)N(iPr)_2$ ('Phosphate-ON™', Cruachem) and 250 μl of 0.4 M tetrazole in CH_3CN (5 min, room temperature), washed with CH_3CN, oxidized with 0.1 M I_2 in pyridine/THF/H_2O 10/10/1 v/v/v (2 min), and detritylated (3% DCA in CH_2Cl_2, 1.5 min). A DMT-nucleoside cyanoethyl phosphoramidite reagent was coupled to the terminal hydroxyl group under standard conditions using tetrazole activation, and the resulting support-bound phosphite triester derivative was sulfurized with a 5% solution of S_8 in pyridine/CS_2 1/1 v/v (45 min, room temperature). (19) The column was then connected to a Cyclone DNA synthesizer and oligonucleotide units were introduced by conventional phosphoramidite chemistry (in the preparation of 26-mer **5**, oxidations were carried out with 0.1 M I_2 in pyridine/acetic acid, 9/1 v/v). After completion of the synthesis the support bound oligomer was released by treatment with concd. NH_4OH (55°C; 2.5 h for dT derivatives and 8 h for mixed base derivatives). The DMT products were separated by RP chromatography and the DMT groups were removed by treatment with 80% aq. acetic acid. The oligomers were then purified by IE HPLC (gradient A, General Methods) and desalted by gel-filtration on a NAP-5 column (Pharmacia).

The chemistry for introducing a phosphorothioate group was initially checked by the preparation and transformations of thymidine 3′-phosphorothioate, Ts. The DMT^+ cation released after loading thymidine on the support corresponded to ~40 μmol thymidine per gram CPG, and the product, Ts (RP HPLC elution time, 12.0 min; Rf TLC 0.3; ^{31}P NMR 43.44 ppm), contained no Tp within the limits of detection (^{31}P NMR spectrum and the RP HPLC chromatogram). For further characterization, a sample of the Ts (35 A_{260} units, ~4 μmol, in 0.5 ml of water) was treated with 40 μl of 0.1 M KI_3 in water at 4°C. Analysis by HPLC after 1 min showed essentially complete conversion to the 3′3′ dimer derivative containing a -OP(O)(O^-)SSP(O)(O^-)O- internucleoside link, $[Ts]_2$; RP HPLC, 15.3 min; TLC, Rf 0.4; ^{31}P NMR 68.84 ppm. The reaction was also carried out (~95% conversion) by treating thymidine 3′-phosphorothioate (4 μmol in 0.5 ml of water) with 0.4 ml of 1 M aqueous $K_3Fe(CN)_6$ at room temperature for 3 h. Reductive cleavage of 1 A_{260} unit of the dimer in 100 μl of the phosphate buffer at room temperature with dithiothreitol (1 mg, 1 h) gave the monomer, Ts, quantitatively.

Oligonucleotide 5′-phosphorothioates

Oligonucleotides were synthesized on a 1 μmole scale on a Milligen/Biosearch Cyclone DNA synthesizer using LCAA CPG supported deoxyribonucleosides loaded through a succinyl linker. When chain elongation was complete, the terminal 5′-hydroxyl group was phosphitilated (5 min) with 150 μL of a 0.1 M solution of ('Phosphate ON™' reagent from Cruachem) in CH_3CN plus 150 μl of 0.5 M tetrazole in CH_3CN. The resulting phosphite was sulfurized by treatment with a 5% solution of S_8 in pyridine/CS_2 (1:1, v/v, 45 min at room temperature). After cleavage of the DMT group (3% DCA in CH_2Cl_2, 1.5 min) the supported oligomer was worked up in a conventional manner.

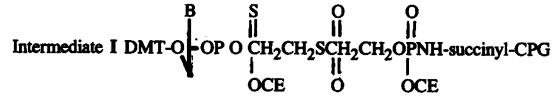
Oxidation of oligonucleotide 3′- and 5′-phosphorothioates

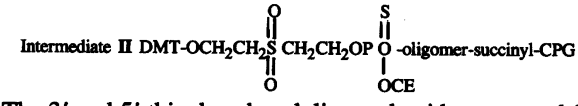
For coupling of terminal thiophosphoryloligonucleotides in absence of a template (Table 1), aqueous $K_3Fe(CN)_6$ (1 μl, 1.0 M) was added to a solution of the oligomer (1 A_{260} unit in 10 μl of water, ~1 mM, at 4°C). After 3 h at this temperature the mixture was desalted (NAP-5 column) and the product was isolated (70–75% yield) by IE HPLC (gradient A). HPLC analysis of products from reaction of 26-mer **5** showed that conversion to the 52-mer, **5A**, was about 50% complete after 30 minutes and about 90% complete after 3 hours. Treatment with DTT (1 mg/ml; 1 h, r.t.) converted **5A** back to **5**.

RESULTS AND DISCUSSION

Synthesis and properties of oligonucleotide phosphorothioate derivatives

To facilitate preparation of 3′- and 5′-thiophosphoryl-oligonucleotides a synthetic procedure was developed that exploits the facile cleavage of sulfones derived from the commercially available 'Phosphate-ON™' reagent. Thus, condensation (20,21) of this phosphoramidite reagent with an amino group of a LCAA CPG support followed by oxidation, detritylation, coupling with a nucleoside cyanoethyl phosphoramidite reagent, and sulfurization afforded intermediate **I**. The 3′-thiophosphoryloligonucleotide was obtained by extending the chain by amidite chemistry and cleaving the oligomer from the support with ammonium hydroxide. For the 5′ derivatives, intermediate **II** was prepared and converted to the modified oligonucleotide by successive treatment with mild acid and ammonium hydroxide.





The 3′ and 5′ thiophosphoryloligonucleotides prepared for this study are listed in Table 1. Yields were comparable to those obtained in synthesizing conventional oligonucleotides of the same size.

Table 1. Oligomers and oxidation products (IE HPLC elution time, min)

	Compounds		Oxidation Products
			[No Template]
1	TTTTTTTTTTs (14.0)[a]	1A	$[TTTTTTTTTTs]_2$ (17.5)[a]
2	ACACCCAATTs (15.6)[b]	2A	$[ACACCCAATTs]_2$ (19.1)[b]
3	$ACACCCAAT_s$ (14.5)[b]	3A	$[ACACCCAATs]_2$ (18.1)[b]
4	$sCTGA_4TGG$ (15.2)[a],(18.3)[b]	4A	$[sCTGA_4TGG]_2$ (18.3)[a],(21.6)[b]5
5	$CGCTGAAG_3CTTCTTCCTTATTGATs$ (25.1)[a]	5A	$[CGCTGAAG_3CTTCTTCCTTATTGATs]_2$ (29.9)[a]
6	sTTTTTTTTTTs (20.0)[a]	6A	sTTTTTTTTTTs (18.0)[a]
		6B	Polymer (broad peak ~27min)[a]
7	$CCAT_4CAGAATTGGGTGT$ (23.0)[b]		
			[Template Directed]
6	[Poly(dA) as template]	6C	Polymer
2 + 4	[7 as template]	8	$ACACCCAATTssCTGA_4TGG$ (20.8)[b]
3 + 4	[7 as template]	9	$ACACCCAATssCTGA_4TGG$ (20.1)[b]

[a]pH 7.0, gradient *A*; [b]pH 12, gradient *B*. Abbreviations: s represents a phosphorothioate group (-OP(O)(OH)S$^-$) at the 3′ end or 5′ end of the oligonucleotide. The formulas $[...s]_2$ and $[s...]_2$ similarly represent dimeric structures in which the monomeric units are linked through 3′3′ or 5′5′ positions by -OP(O)(O$^-$)SSP(O)(O$^-$)O- groups. All nucleotides are deoxyribonucleotides.

Oxidation in absence of templates

Both KI_3 and $K_3Fe(CN)_6$ were found to be effective oxidants for converting oligonucleotide monophosphorothioates to 'dimeric' 'S-S' derivatives. They differ, however, in reactivity. Couplings induced by $K_3Fe(CN)_6$ in absence of templates are relatively slow. They are conveniently carried out using a large excess of oxidant and long reaction times (several hours). Reactions with KI_3 are much faster; however, oxidative desulfurization of thiophosphoryl groups also occurs. Conditions favoring high coupling yields in this case are a low ratio of oxidant to sulfur (1−3 equivalents of KI_3), low temperature, and a short reaction time (a few minutes).

Formulas and HPLC data for coupling products (**1A**−**5A**) obtained by oxidizing compounds **1**−**5** with $K_3Fe(CN)_6$ are listed in Table 1. The reactions proceeded well, even in the case of a 26-mer (**5**). Dithiothreitol (DTT) readily cleaved the dimeric products to the monomeric oligonucleotide phosphorothioates.

It is noteworthy that thymidylate oligomer **1A**, in which one decamer segment has a 5′ to 3′ orientation and the other a 3′ to 5′ orientation, forms a relatively stable 1:1 complex with poly(dA). The Tm value, 29.5°C (observed at both 260 and 282 nm, 0.1 M Na^+), is well above that for the corresponding complex of monomer unit **1** (21.5°C), and the hyperchromicity on melting (30%) is relatively high, indicative of organize base stacking for all the thymidine units. We suggest as an attractive possibility a duplex structure in which molecules of **1A** are aligned along a poly(dA) strand with one block of thymidylate units oriented parallel, and the other antiparallel, to the poly(dA) strand. This suggestion accords with the report that oligo(dT)/oligo(dA) segments form hydrogen bonded structures with a parallel strand orientation when appropriately constrained by geometry. (22)

The products from oxidation of the 3′,5′-bisphosphorothioate oligomer (**6**) were more complex. As shown by curve A in Figure 1, decamer **6** forms a complex with poly(dA) in aqueous solution. The Tm value is ~15°C, somewhat lower than that for TTTTTTTTTTs/poly(dA). For curve B (Figure 1), **6** was first oxidized with $K_3Fe(CN)_6$, then the products were mixed with poly(dA) for a Tm measurement. The 0−40°C region of the melting curve in this case is similar to that observed on heating solutions of free poly(dA); there is no break indicative of formation of a complex either with products of oxidation or with residual **6**. However, a transition (Tm 50°C) exhibiting relatively low hyperchromicity is found in the 40−60°C region. This transition can be attributed to dissociation of complexes derived from poly(dA) and polymers possessing -sTTTTTTTTTTs-units randomly linked head-to-head, head-to-tail, tail-to-head, and tail-to-tail).

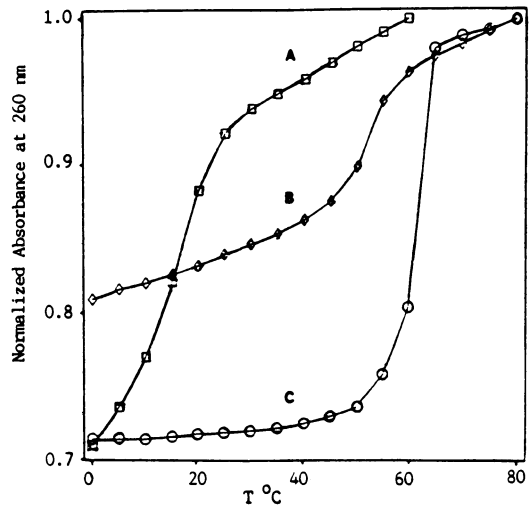


Figure 1. Thermal dissociation curves of poly(dA) + compound **6** or its oxidation products in phosphate buffer, pH 7.05, 0.1 Na^+. A: compound **6** (0.13 mM in dT) + poly(dA) (0.13 mM in dA). B: To 1 ml of a solution of **6** (0.13 mM in dT units) in the standard buffer solution at 0°C was added 10 μl of 0.1 M aq. $K_3Fe(CN)_6$; the mixture was kept at 4°C for 1 day, desalted, concentrated, and then taken up in 1 ml of buffer containing an equivalent of poly(dA) for the heating experiment (see Methods section). C: The oxidation was carried out as in B except that the equivalent of poly(dA) was present during reaction with $K_3Fe(CN)_6$.

Analysis by IE HPLC of the products of oxidation of **6** by $K_3Fe(CN)_6$ revealed two major products with about equal absorbance. One eluted as a sharp peak at 18 min, somewhat earlier than **6** would have eluted (20 min). The other appeared as a broad peak centered ~27 min. These results from the HPLC analysis and the heating experiments, taken together, indicate that

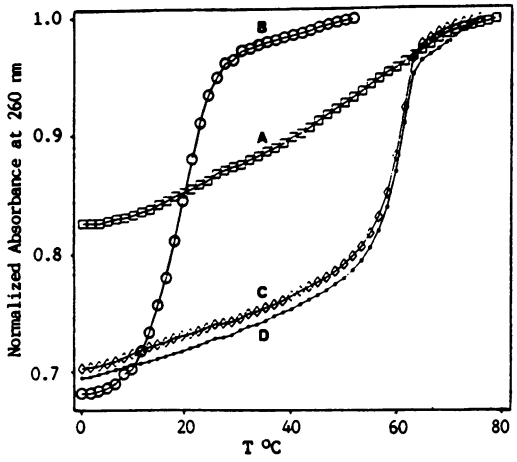


Figure 2. Thermal dissociation curves for oxidation of **6** with KI_3. **A:** An aqueous solution of KI_3 (100 μl, 0.2 mM) was added to a solution of compound **6** (1.0 ml, 0.06 mM in dT units) in the standard buffer at 0°C; after 3 min one half of the solution was desalted by gel filtration, concentrated, and mixed with an equivalent of poly(dA) in buffer for the Tm measurement. **B:** The other half portion from experiment A was treated with dithiothreitol (1 mg) and warmed at 55°C for 30 min; after cooling and desalting the thermal experiment was carried out as before. **C:** The nucleotide products from the heating experiment in B were collected by gel filtration and oxidized with $K_3Fe(CN)_6$ (1 μl of 0.1 M solution, 4°C, 15 min); then the solution was desalted and taken up in buffer for the Tm measurement. **D:** Oxidation was carried out for 2 min at 0°C in a solution (phosphate buffer, pH 7.0, 0.1 M Na^+) containing compound **6** (6 μM, 0.06 mM in dT units) and poly(dA) (0.06 mM in dA units) and KI_3 (12 μM); then the mixture was desalted, concentrated and dissolved in buffer for the Tm measurement.

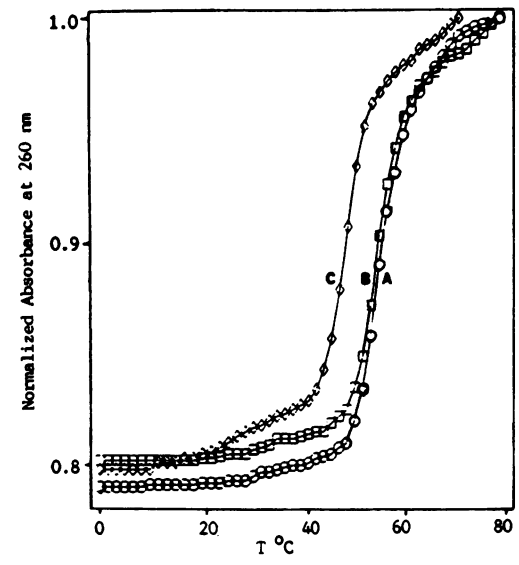


Figure 3. Thermal dissociation curves for oxidative ligation of ACACCCAATTs (**2**) or ACACCCAATs (**3**) with $sCTGA_4TGG$ (**4**) on template **7**. **A:** Compounds **2**, **4**, and **7** were mixed in phosphate buffer (0.1 M Na^+) at 0°C to give solutions 0.2 μM in each; then 1 μl of 1.0 M $K_3Fe(CN)$ was added. The mixture was shaken and kept at 0°C for 5 min. A small portion was used for IE HPLC. The remainder was desalted on a NAP-5 column, concentrated in vacuo, and redissolved in the standard buffer for the Tm measurement. **B:** The ligation and work-up were carried out as in A except that 5 μl of 2 mM KI_3 was used as oxidant and the reaction time was 2 min. **C:** The ligation was carried out with **3**, **4**, and template **7** (each 2μM) as in B, using KI_3 as oxidant.

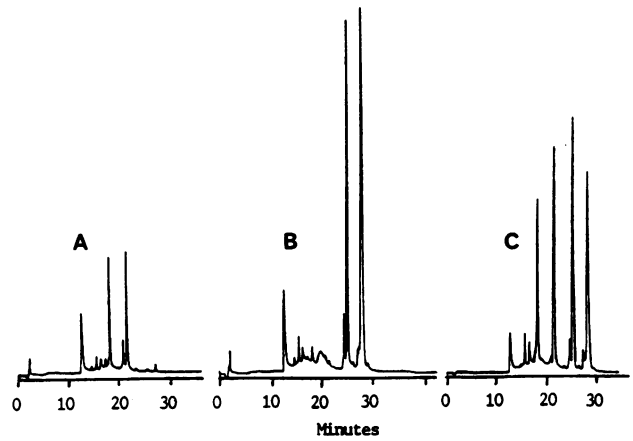


Figure 4. Ion exchange HPLC profiles, pH 12.0, gradient *C*. **A:** Decamers **2** and **4**, 2μM each in 1 ml of buffer, were treated with 1 μl of 1.0 M $K_3Fe(CN)_6$; after ~20 min at 0°C, a sample was subjected to IE HPLC; the principle peaks correspond to ferricyanide (13 min), **2** (18.5 min), and **4** (22 min), respectively. **B:** Products from oxidation of **2** and **4** with $K_3Fe(CN)_6$ in presence of template **7** (products from experiment in Figure 3A, before heating); the major peaks correspond to ferricyanide, **8** (25 min), and **7** (28 min), respectively; bands in the 15–23 min range are primarily non-nucleotidic. **C:** The reaction conditions were the same as in Figures 4B and 3A except 1/3 rather than one equivalent of template **7** was used; the major peaks correspond to **2**, **3**, **8**, template **7**.

oxidation with $K_3Fe(CN)_6$ converts **6** about equally to a low molecular weight product that binds poorly if at all to poly(dA) and to a mixture of polymers that bind moderately well to poly(dA).

When KI_3 was used as the oxidant, only the low molecular weight product was obtained, as shown both by IE HPLC (elution time 18 min; no peak corresponding to polymers) and by heating with poly(dA). The melting curve for the latter experiment (curve A, Figure 2) is an essentially featureless line similar to the lower portion of the curve in Figure 1B. Although a definitive structure for this product has not been established, the evidence points strongly to a cyclic decanucleotide containing a -OP(O)(O^-)-SSP(O)(O^-)O- bridge (formula for **6A**, Table 1). (23) In support of a disulfide type bridge structure, reduction of the product from experiment A, Figure 2, with dithiothreitol yielded a compound exhibiting the properties of decamer **6** (curve B, Figure 2).

Oxidation in presence of templates

Polymerization. When the oxidation of **6** with $K_3Fe(CN)_6$ was carried out in the presence of poly(dA), a very different melting profile was obtained. As shown by curve C in Figure 1 a strong, sharp break is found in the high temperature region (Tm 61°C). The curve resembles that for poly(dT)/poly(dA) (Tm 68°C, 0.1 M NaCl). (24) Similarly, after addition of poly(dA) to a solution containing the phosphorothioate derivative generated by reduction of **6A** (see curve B, Figure 2), oxidation with $K_3Fe(CN)_6$ afforded a 'high melting' complex (curve C, Figure 2). This reaction was carried out under conditions for which little if any coupling occurs in absence of poly(dA).

As evident from a comparison of curves A [for oxidation without poly(dA)] and D [for oxidation in presence of poly(dA)] in Figure 2, the course of the reaction of **6** with KI_3 is likewise markedly altered by the presence of a template. Just as in the template controlled oxidation with $K_3Fe(CN)_6$, a polymer that

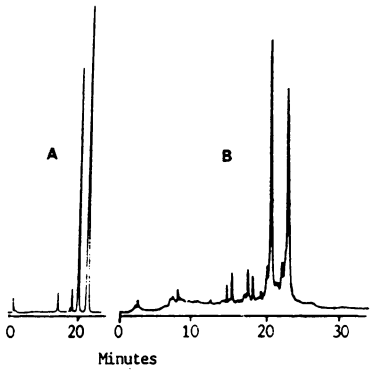


Figure 5. A: IE HPLC (pH 12.0, gradient *B*) of products from ligation of **3** + **4** on template **7** using KI_3; see experiment for Figure 3C. **B:** Products from the equilibration reaction described in Figure 6C were cooled to room temperature, stored overnight, and subjected to IE HPLC at pH 12.0, gradient *B*. Peaks at 20 and 23 min correspond to **9** and **7**, respectively.

forms a 'high melting' complex with poly(dA) is obtained when the oxidation is carried out in presence of poly(dA).

We conclude from these experiments that poly(dA) serves as a template to align oligonucleotide 3′5′-bisphosphorothioate molecules in a head to tail fashion in proximity for efficient, rapid, and orientationally selective polymerization.

Coupling of mixed base oligonucleotides. As a test of utility of this chemistry in joining oligonucleotides containing mixed bases, we investigated the oxidative coupling of ACACCCAATTs (**2**) to sCTGAAATGG (**4**). These oligomers form a complex with the complementary strand, CCATTTTCAGAATTGGGTGT (**7**), that dissociates with a Tm value of 29°C (0.1 M Na^+). Under mild conditions in dilute solution (0°C, 5 min, 2 μM oligomer) in absence of a template, essentially no coupling occurs when a solution of **2** + **4** is treated with $K_3Fe(CN)_6$ (see HPLC profile in Figure 4A). However, when the oxidation is conducted in the presence of template **7**, coupling is essentially complete under these conditions (see HPLC profile in Figure 4B). When the template is the limiting reagent, limited coupling occurs and, as expected, peaks corresponding to the coupling product and unreacted **2**, **4**, and **7** are observed (HPLC profile C in Figure 4).

The melting curve for the heteroduplex formed in the template directed reaction provides strong evidence that the oxidation product indeed comprises two linked oligonucleotide blocks complementary in sequence to oligomer **7** (Curve A, Figure 3). The Tm value (55°C) is 27°C higher than that for the complex of the non-ligated reactants and near that for the natural type duplex possessing the the same nucleotide sequence (Tm 60°C (25). The data in Figure 3, curve B, demonstrate that KI_3, like $K_3Fe(CN)_6$, is an effective agent for template directed oxidative coupling of these oligomers.

In this system (**2**+**4**+**7**) the bases in the oligomer blocks complement the bases in the template throughout; ligation introduces a diphosphorothioate bridge in one strand opposite a phosphodiester link in the other. We selected the reaction of ACACCCAATs (**3**) with **4** in the presence of **7** to probe structural requirements in the vicinity of the linkage site. Relative to compound **2**, compound **3** lacks one nucleotide at the 3′ end. Ligation in this case would position a diphosphorothioate bridge opposite a phosphodiester-nucleoside-phosphodiester link in the template.

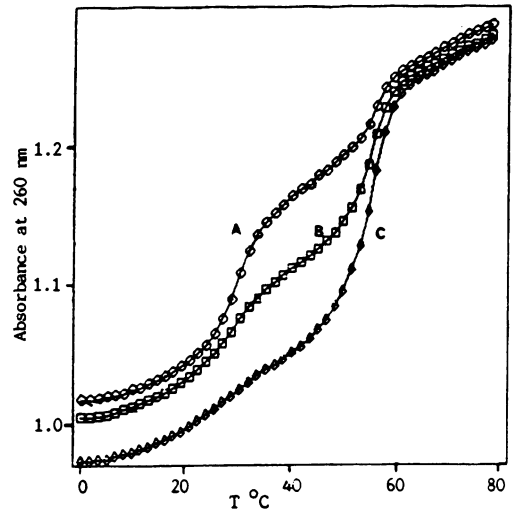


Figure 6. A: Thermal dissociation curve for **2A** + **4A** + **7**, each 2 μM in standard buffer (0.1 M Na^+). **B:** A catalytic amount of **2** (~40 nM) was added to the solution from A while at 80°; then the solution was cooled, kept for 30 min at 0°C, and used for the the Tm measurement. **C:** The solution from B was cooled, kept at 0°C for 30 min, and used again for a Tm measurement.

Melting data (Figure 3C) and HPLC profiles (see Figure 5E for products of KI_3 oxidation) show that oligomers **3** and **4** *do* couple when oxidized in the presence of a template containing an extra nucleotide at the juncture site. The template requirements for this chemical coupling reaction are therefore less stringent than for enzymatic ligation.

Recombination of oligomers

Oligonucleotide derivatives containing these S−S bridges are stable in aqueous solutions, even on warming to 80°C at pH 7 or on standing at pH 12 for several hours at room temperature. We found, however, that under appropriate conditions such oligomers undergo a recombination reaction controllable by a template in the solution. This process was observed during investigation of the interaction of the S-S derivatives, **2A** and **4A**, with template **7**. When equimolar amounts of **2A**, **4A**, and **7** were heated, melting curve A, Figure 6, was obtained. The major break, which occurs in the low temperature range (Tm ~30°C), may be attributed to dissociation of complexes in which each of the 'dimer blocks' is aligned on two different molecules of the template. The break at higher temperature corresponds to dissociation of the complex formed from **8** and **7**. On the assumption that residual phosphorothioate catalyzed an exchange of the two oligomer blocks, a small sample of **2** (~5% of the amount of **2A**) was added to facilitate the reaction. Two cycles of cooling and reheating then led to curves B and C, respectively. These profiles show that, in the process of heating and cooling, compounds **2** and **4** were converted to an oligomer that formed a more stable complex with **7**. The plausible assumption that the new oligomer is **8**, a product of recombination, is supported both by the Tm value for the complex formed with **7** (Tm 55°C, in agreement with the value observed in Figure 3A) and by the IE HPLC profiles for the products obtained from the final heating experiment (see E, Figure 5B). As shown by the chromatogram, oligomers **2** (elution time 19.1 min) and **4** (21.6 min) were largely converted to an oligomer with the properties of **8** (20.8 min). The extent of the conversion far exceeded the statistical limit of 50% expected for a random redistribution reaction. We therefore

conclude that for these oligonucleotide derivatives, recombination at diphosphorothioate bridges (induced by free phosphorothioate), as well as direct oxidative ligation of terminal phosphorothioates, can be directed by base pairing interactions with a template.

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ABBREVIATIONS

TEAA, triethylammonium acetate; IE, ion exchange; RP, reversed phase; Ts, thymidine 3′-phosphorothionate; $[Ts]_2$· a dimer derivative of Ts containing an internucleoside -OP(O)(O^-)SSP(O)(O^-)O- link. All oligomers are deoxyribonucleotide derivatives.

REFERENCES

1. Naylor, R., & Gilham, P.T. (1966) Biochemistry, **5**, 2722–2728.
2. Gellert, M. (1967) Proc. Natl. Acad. Sci. USA, **57**, 148–145.
3. Olivera, B.M., & Lehman, I.R. (1967) Proc. Natl. Acad. Sci. USA, **57**, 1426–1433.
4. Zimmerman, S.B., Little, J.W., Oshinsky, C.K., & Gellert (1967) Proc. Natl. Acad. Sci. USA, **57**, 1841–1848.
5. Gefter, M., Becker, A, & Hurwitz, J. (1967) Proc. Natl. Acad. Sci. USA, **58**, 240–247.
6. Weiss, B., Live, T.R., & Richardson, C.C. (1967) Fed. Proc. **26**, 395.
7. Becker, A., Gefter, M., & Hurwitz (1967) Fed. Proc. **26**, 395 (1967).
8. Gupta, N.K., Ohtsuka, E.; Weber, K., Chang, S.H., & Khorana, H.G. (1968) Proc. Natl. Acad. Sci. USA, **60**, 285–292.
9. Kanaya, R., & Yanagawa, H. (1986) Biochemistry, **25**, 7423–7430.
10. Luebke, K.J., & Dervan (1991) J. Am. Chem. Soc., **113**, 7447–7448, (1992) Nucleic Acids Res., **20**, 3005–3009.
11. Sokolova, N.I., Ashirbekova, D.T., Dolinnaya, N.G., & Shabarova, Z.A. (1988) FEBS Letters, **232**, 153–155.
12. Shabarova, Z.A. (1988) Biochemie, **70**, 1323–1334.
13. Lohrmann, R., & Orgel, L.E. (1978) Tetrahedron, **34**, 853–855.
14. Purmal, A.A., Shabarova, Z.A., & Gumport, R.I. (1992) Nucleic Acids Res., **20**, 3713–3719.
15. Kool, E.T. (1991) J. Am. Chem. Soc., **113**, 6265–6266.
16. Ashley, G.W., & Kushlan, D.M. (1991) Biochemistry, **30**, 2927–2933.
17. Eckstein, F. (1966) J. Am. Chem. Soc. **88**, 4292–4294.
18. Wu, T., & Orgel, L.E. (1991) J. Mol. Evol., **32**, 274–277.
19. Burgers, P.M.J., & Eckstein, F. (1978) Tetrahedron Lett., **40**, 3835–3838. Connolly, B.A., Potter, B.V.L., Eckstein, F., Pingoud, A., & Grotjahn, L. (1984) Biochemistry, **23**, 3443–3453.
20. Gryaznov, S.M., & Letsinger, R.L. (1992) Nucleic Acids Res., **20**, 3403–3409.
21. Gryaznov, S.M., & Letsinger, R.L., Tetrahedron Lett., in press.
22. Van de Sande, J.J., Ramsing, N.B, Germann, M.K., Elhorst, W., Kalisch, B.W., Kitzing, E.v., Pon, R.T., Clegg, R.S., & Jovin, T.M. (1988) Science, **241**, 551–557.
23. The difference in product distribution observed for the two oxidants may reflect different reaction mechanisms. Oxidative coupling of phosphorothioates induced by $K_3Fe(CN)_6$ probably involves reaction of two P-S· radicals [see Neumann, H., Steinberg, I.Z., & Katchalski, E. (1965), J. Am. Chem. Soc., **87**, 3841–3848]. In the oxidation with KI_3, P-SI intermediates may also form and react directly with the anionic P-S^- groups. This process could favor intramolecular coupling relative to intermolecular coupling in dilute solution.
24. Riley, M., Maling, B., & Chamberlin, M. (1966), J. Mol. Biol., **20**, 359–389.
25. Letsinger, R.L., Zhang, G., Sun, D.K., Ikeuchi, T., and Sarin, P.S. (1989) Proc. Natl. Acad. Sci. USA, **86**, 6553–6556.