Chemical Ligation of Oligonucleotides in the Presence and Absence of a Template

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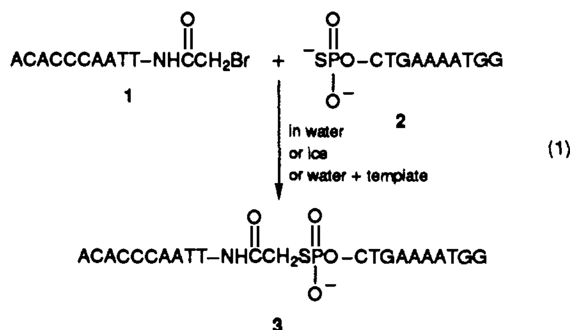
Several methods have been reported for covalently linking oligonucleotide blocks in aqueous media.[1] All utilize "activating agents" to initiate the reaction and require the presence of a complementary oligonucleotide template for efficient and selective coupling. As in enzymatic ligation, the template serves to bring the designated termini of the polymers into position favorable for reaction. For the construction of complex polynucleotide systems, synthetic methodology enabling efficient coupling of oligonucleotide blocks in the absence, as well as in the presence, of a template could be helpful. We describe such a procedure here. The ligation, indicated in eq 1 for oligomers **1**[2] and **2**,[1k] exploits the facile reaction of a phosphorothioate with an α-halo acyl derivative.[3] Depending on the situation, coupling of oligonucleotides can be carried out advantageously (a) in aqueous solution, (b) in the frozen state in ice, or (c) in aqueous solution in the presence of a template.



In an initial experiment, 1.0 mL of a solution (pH 7.05, 15 mM phosphate, 85 mM NaCl) containing compounds **1** (0.39 A_{260} units, 4 μM) and **2** (0.41 A_{260} units, 4 μM) was prepared and kept at 0 °C for 5 days, then warmed to 50 °C for 2.5 h, and finally frozen and stored at −18 °C for an additional 5 days. Analysis by IE HPLC[4] of samples after 2 and 48 h showed formation of a slower eluting product, oligomer **3**, in yields of about 25% and 80%, respectively. No significant change was observed after the additional 3 days at 0 °C or warming at 50 °C; however, the reaction did proceed further in the frozen state, affording a high conversion to **3** within 5 days (Figure 1). The enhanced extent of reaction in the ice matrix may be attributed to the high local concentration of the oligonucleotide reactants within the cavities in the ice.[5]

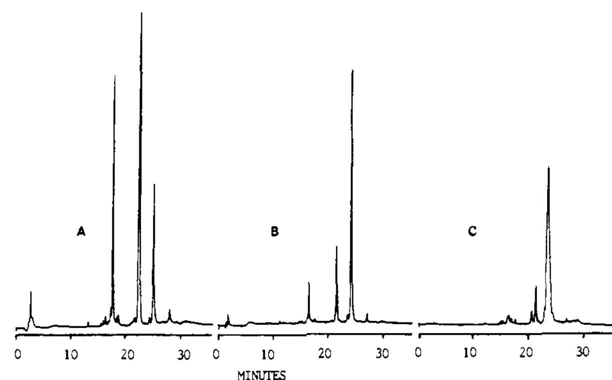


Figure 1. IE HPLC of products from experiment 1: (A) after 2 h in solution at 0 °C; (B) after 2 days at 0 °C; and (C) after the final step in which the solution was frozen and stored at −18 °C for 5 days. The peaks at ~17, 21, and 24 min correspond to compounds **1**, **2**, and **3**, respectively.

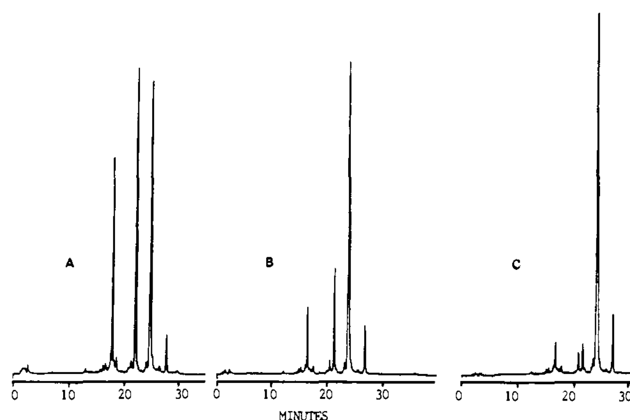


Figure 2. IE HPLC of products from experiment 2 (frozen, −18 °C throughout) after (A) 5 h; (B) 2 days; and (C) 5 days. The peaks at ~17, 21, and 24 min correspond to **1**, **2**, and **3**; that at 27 min corresponds to the dimer derivative of **2** produced by oxidation by air.

In light of this result, an equimolar mixture of **1** and **2** (2 μM each) in the same buffer was directly frozen and held at −18 °C. The HPLC profiles obtained from samples after 5 h and daily thereafter show progression to give a high yield of **3** in 5 days (see Figure 2 for representative data).

Data for coupling **1** and **2** in solution in the presence of a complementary oligonucleotide template (CCATTTTCAGAATTGGGTGT, compound **4**) are presented in Figure 3. The system was the same as in the first experiment except that template **4** was also present (4 μM). In this case the reaction proceeded to >90% completion within 20 min and was essentially complete within 2 h.

(1) (a) Naylor, R.; Gilham, P. T. *Biochemistry* **1966**, *5*, 2722–2728. (b) Sokolova, N. I.; Ashirbekova, D. T.; Dolinnaya, N. G.; Shabarova, Z. A. *FEBS Lett.* **1988**, *232*, 153–155. (c) Shabarova, Z. A. *Biochimie* **1988**, *70*, 1323–1334. (d) Chu, B. C. F.; Orgel, L. E. *Nucleic Acids Res.* **1988**, *16*, 3671–3691. (e) Kool, E. T. *J. Am. Chem. Soc.* **1991**, *113*, 6265–6266. (f) Ashley, G. W.; Kushlan, D. M. *Biochemistry* **1991**, *30*, 2927–2933. (g) Luebke, K. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1991**, *113*, 7447–7448. (h) Luebke, K. J.; Dervan, P. B. *Nucleic Acids Res.* **1992**, *20*, 3005–3009. (i) Prakask, G.; Kool, E. T. *J. Am. Chem. Soc.* **1992**, *114*, 3523–3527. (j) Purmal, A. A.; Shabarova, Z. A.; Gumport, R. I. *Nucleic Acids Res.* **1992**, *20*, 3713–3719. (k) Gryaznov, S. M.; Letsinger, R. L. *Nucleic Acids Res.*, in press.

(2) Compound **1** has a 3′-[(bromoacetyl)amino]-3′-deoxythymidine unit at the 3′-terminus. For preparation of **1**, 15 μL of 0.4 M aqueous *N*-succinimidyl bromoacetate (Calbiochem) was added to 4.9 A_{260} units of the 3′-amino deoxyribooligonucleotide precursor, ACACCCAATT-NH_2 [prepared by the method described by Gryaznov and Letsinger: Gryaznov, S. M.; Letsinger, R. L. *Nucleic Acids Res.* **1992**, *20*, 3403–3409] in 10 μL of 0.2 M sodium borate buffer at room temperature. After 35 min the mixture was diluted (0.5 mL of H_2O), desalted by gel filtration on a NAP-5 column (Pharmacia), purified by RP HPLC, and again desalted, giving 4 A_{260} units of **1**; elution time, RP HPLC, 17.4 min; IE HPLC, 17.4 min (ref 4).

(3) Reaction of a thiophosphoryl oligonucleotide with a (haloacetyl)amino aromatic derivative in DMSO/H_2O has been employed in preparing dye-oligonucleotide conjugates: (a) Thuong, N. T.; Chassignol, M. *Tetrahedron Lett.* **1987**, *28*, 4157–4160. (b) Francois, J.-C.; Saison-Behmoaras, T.; Barbier, C.; Chassignol, M.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9702–9706.

(4) All IE HPLC was carried out on a Dionex Omni Pak NA100 4 × 250 mm column at pH 12 (10 mM NaOH) with a 2%/min gradient of 1.0 M NaCl in 10 mM NaOH. For RP HPLC a Hypersil ODS column, 4.6 × 200 mm, was used with a 1%/min gradient of acetonitrile in 0.03 M triethylammonium acetate buffer, pH 7.0.

(5) For some other reactions carried out in an ice matrix, see: (a) Beukers, R.; Ylstra, J.; Berends, W. *Recl. Trav. Chim. Pays-Bas* **1958**, *77*, 729–732. (b) Letsinger, R. L.; Ramsay, O. B.; McCain, J. H. *J. Am. Chem. Soc.* **1965**, *87*, 2945–2953.

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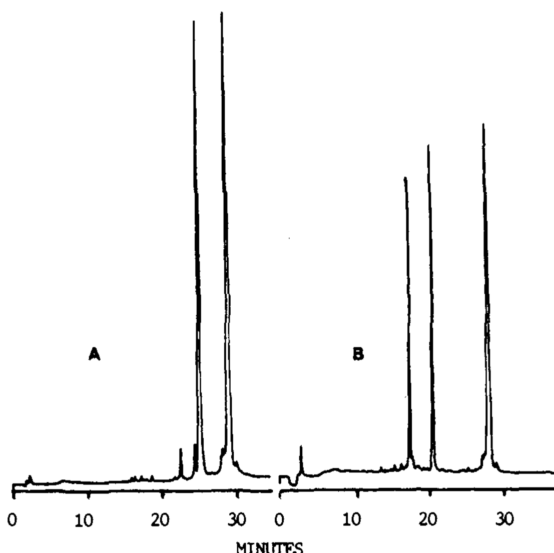

Figure 3. (A) IE HPLC of products from reaction of **1** and **2** in the presence of template **4** at 0 °C, after 2 h. The major peaks correspond to coupling product **3** and template **4**. Note that **1** (17 min) has been almost completely consumed. (B) Same products after treatment with KI_3 followed by DTT; note that **3** has been replaced by two oligonucleotide cleavage products, eluting at 18 and 22 min.

The structure assigned to **3** is supported by the properties of a model compound (T–NHC(O)CH_2–SP(O)(O^-)O–T, prepared in solution on a larger scale than used for **3**),[6] by the mobility of **3** on gel electrophoresis[7] (R_m 0.58, compared to R_m 0.89, 0.95, and 0.61 for **1**, **2**, and **4**, respectively), and by the stability of the complex formed with the complementary oligonucleotide **4**. The T_m value, 56 °C in 0.1 M NaCl, approaches that of the complex formed from the corresponding all-phosphodiester 20-mer and **4** (60 °C[8]) and differs signficantly from values for complexes formed from **1** or **2** with **4** (37 and 31 °C). In addition, the internucleotide –NH(CO)CH_2SP(O)(O^-)– link was found to cleave selectively on oxidation with KI_3[9] (Figure 3).

In summary, this chemistry provides a convenient means for selectively and irreversibly coupling oligonucleotides in aqueous solution in the range of 4 μM oligomer concentration or greater. The products are stable in neutral solution, and for a few hours even at pH 12 at room temperature. At concentrations below 1 μM, the rate in the liquid phase becomes extremely slow; however, the reactions can be carried to near completion in the frozen state. The rate of coupling is markedly accelerated by the presence of a complementary oligonucleotide template. These properties, considered together, indicate that this coupling chemistry could have potential in the design of chemical amplification systems and in *in situ* ligation in antisense applications as well as in building complex structures from oligonucleotide blocks.

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(6) Retention time, RP HPLC 10.5 min; FAB+ mass spectrum, M + H^+ 620, M + Na^+ 642; ^{31}P NMR, δ in D_2O, 18.7 ppm (characteristic for the alkylthiophosphate group; see: Mag, M.; Luking, S.; Engels, J. W. *Nucleic Acids Res.* **1991**, *19*, 1437–1441).

(7) R_m values are relative to bromophenol blue in a 20% polyacrylamide/5% bis acrylamide gel.

(8) Letsinger, R. L.; Zhang, G.; Sun, D. K.; Ikeuchi, T.; Sarin, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6553–6556.

(9) The duplex containing **3** and **4** (0.3 A_{260} units each) in 100 μL of water was treated with 100 μL of 0.2 M aqueous KI_3 for 15 min at 50 °C; then 10 μL of 1 M aqueous DTT was added. After 5 min the mixture was desalted on a NAP-5 column and analyzed by IE HPLC.