Template-Free Segmental Synthesis of Oligonucleotides Containing Nonnative Linkages

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Protected oligonucleotides containing 3′-alkyl carboxylic acids or 3′-alkylamines were obtained from photolabile solid-phase synthesis supports (**1** and **4**). Protected oligonucleotides containing 5′-alkylamines and 3′-hydroxyl groups were obtained using a photolabile solid-phase synthesis support (**2**) and a commercially available phosphoramidite reagent (**3**). Depending upon the source of alkylamine-containing oligonucleotide, the segments were coupled under mild conditions to form products containing either 5′−3′ or 3′−3′ linkages in good yield and high purity. Oligonucleotides as long as 40 nucleotides were prepared, and coupling yields of protected biopolymer segments were independent of length over the range examined. This method is particularly well suited for the convergent synthesis of oligonucleotides containing nonnative linkages and should be useful for the rapid assembly of modified biopolymers that are useful in biochemical studies.

Oligonucleotide synthesis is readily accomplished using automated methods on solid-phase supports. Automated synthesis is particularly useful for those biopolymers composed of native nucleotides and/or nonnative constituents that are stable to routine synthesis and deprotection conditions, which include protic acid and base, respectively.[1] Solid-phase oligonucleotide synthesis is most often carried out in a linear fashion whereby one nucleotide is added at a time. Provided satisfactory coupling yields of segments could be obtained, the convergent nature of segmental synthesis would be an inherently more efficient process. Segmental synthesis could be particularly useful in studies where the modification of one domain of a biopolymer is of interest, such as in the search for new catalysts and/or nucleic acids capable of molecular recognition.[2] Segmental synthesis could also prove useful in large-scale oligonucleotide production, where cost and purification are important issues. We wish to report initial results concerning the development of a general method for segmental synthesis of oligonucleotides containing nonnative linkages.

Prior to the advent of modern oligonucleotide synthesis methods, long oligonucleotides were prepared by coupling fully deprotected biopolymers in solution. In their ground breaking synthesis of the gene for the precursor of a tyrosine suppressor tRNA, Khorana et al. enzymatically coupled templated oligonucleotide segments.[3] The segments themselves were synthesized chemically in solution, with the reaction between an undecamer and a tetramer describing the coupling between the longest segments. One general advantage of synthesizing longer oligonucleotides by coupling shorter oligomers is greater ease of purification, since failure sequences and full-length product will differ more significantly in length than comparable polymers prepared one nucleotide at a time.[4] The "blockmer" approach for solid-phase oligonucleotide synthesis has been developed and is particularly advantageous for preparing nucleic acids containing phosphorothioates using dinucleotide blocks.[5] There are examples where as many as eight trinucleotide phosphoramidites have been incorporated during solid-phase oligonucleotide synthesis, but to our knowledge components longer than three nucleotides have not been utilized.[6] Reductive amination has been used to couple longer oligonucleotides postsynthetically to produce functional ribozymes, and a variety of chemical agents have been used to assemble longer oligonucleotides.[7] However, these approaches typically (but not always) require complementary sequences as templates and are not routinely employed for synthesizing oligonucleotides.[8,9] In contrast, segmental peptide synthesis was developed more than one decade ago, and various modifications of native peptide ligation have appeared more recently.[10,11] The latter method has proven to be particularly effective for preparing proteins and their modified forms.[12]

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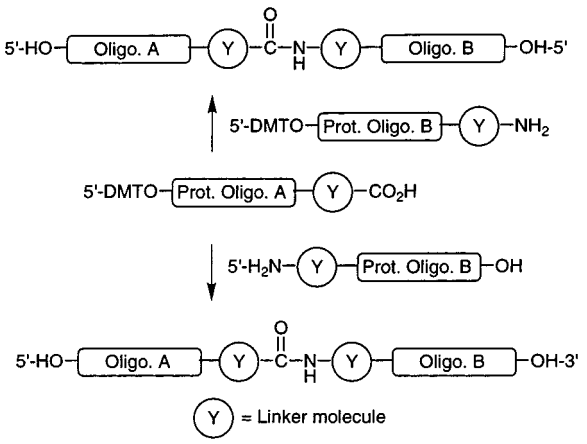
Scheme 1



Our research group has developed a variety of solid-phase synthesis supports and phosphoramidites that enable the unmasking of a single electrophile or nucleophile in an otherwise fully protected oligonucleotide. These reagents have been used to prepare oligonucleotide conjugates involving a variety of small molecules and peptides containing as many as nine amino acids in high yield under mild conditions.[13−17] This approach has been extended to the solution-phase segmental coupling of protected oligonucleotides via nonnative amide bonds (Scheme 1). The method does not require a template and can be used to assemble oligonucleotide segments of either polarity.

Results and Discussion

Solution-Phase Segment Coupling of Protected Oligonucleotides Containing 3′-Alkyl Carboxylic Acids with Protected Oligonucleotides Containing 5′-Alkylamines. Protected oligonucleotides containing the appropriate functional groups were prepared using photolabile solid-phase synthesis supports (**1** and **2**). Protected oligonucleotides containing 3′-alkyl carboxylic acids obtained from **1** have been used previously to prepare oligonucleotide conjugates in solution.[13] Photolysis of protected oligonucleotides prepared on **2** yield products containing 3′-hydroxyl groups.[18] Coupling **3** at the 5′-termini of oligonucleotides synthesized on **2** enables us to obtain solutions of protected oligonucleotides containing 5′-alkylamines and 3′-hydroxyl groups, which could be conjugated to the protected oligonucleotides containing 3′-carboxylic acids. The concentrations of oligonucleotide solutions containing 3′-carboxylic acids (**5**−**8**) were established by treating aliquots with *p*-toluenesulfonic acid and quantitating the dimethoxytrityl cations released.[14] Concentrations of oligonucleotide solutions containing **9** were determined in a slightly more

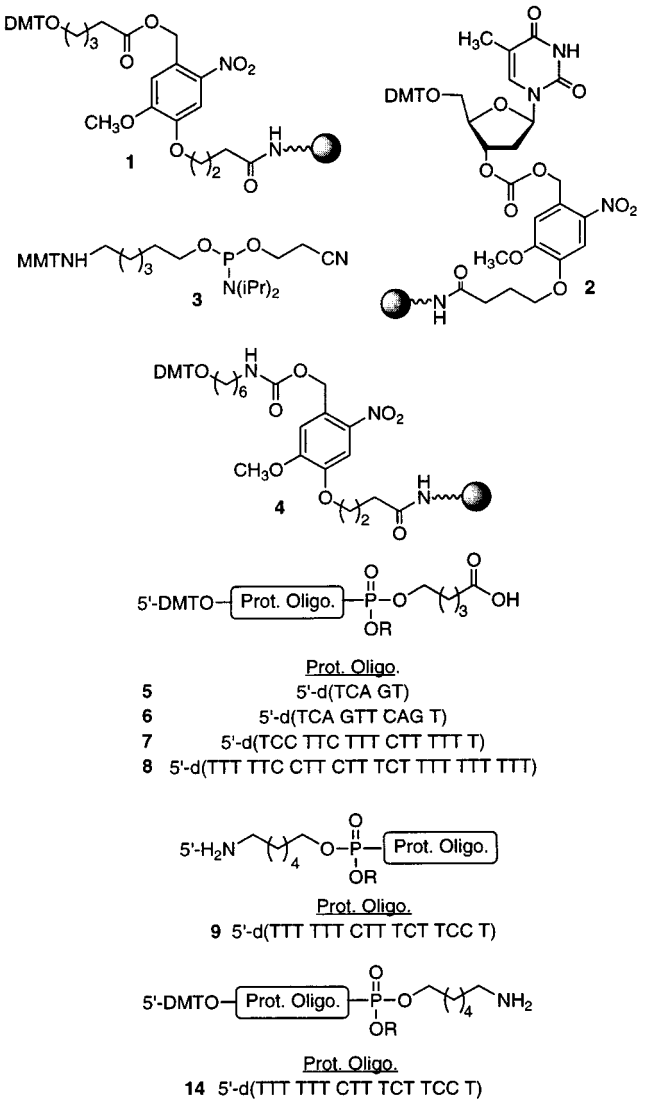


Figure 1.

complicated manner. Small scale irradiations of protected oligonucleotides on **2** were carried out, and the photolysis yields were determined as above. Large scale photolyses were then carried out on the same batch of synthetic material, which was detritylated while on the solid-phase support prior to photolysis. We have found that the photolysis yield is scalable in similar systems,[17] and it was assumed that the small and large scale photolyses proceeded in comparable yield. Moreover, this assumption was verified by treating the crude photolyzate with concentrated aqueous ammonia and determining the photolysis yields by measuring the A_{260} of the sample.

Coupling reactions were carried out for 2 h at room temperature using 2 equiv of carboxylic acid containing oligonucleotides relative to 5′-alkylamine substrate in the presence of PyBOP activating agent (5 equiv) and diisopropylethylamine (5 equiv). Products (**10**−**13**) were isolated by denaturing polyacrylamide gel electrophoresis, following detritylation and ammoniacal deprotection. All products were characterized by ESI-MS.[19] Products **12** and **13** contained small amounts of oligonucleotides lacking either thymidine or deoxycytidine that were attributable to deletions in the respective 3′-carboxylic

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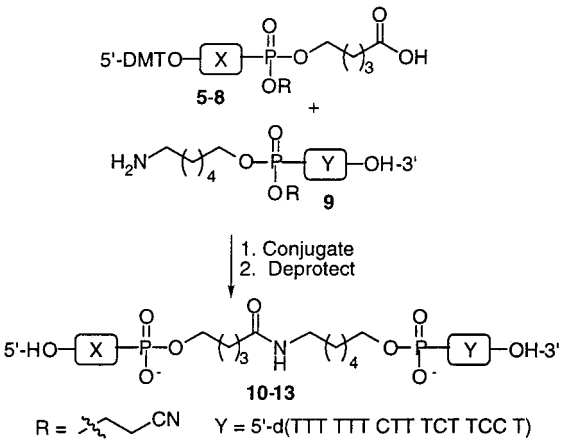
(19) See Supporting Information.

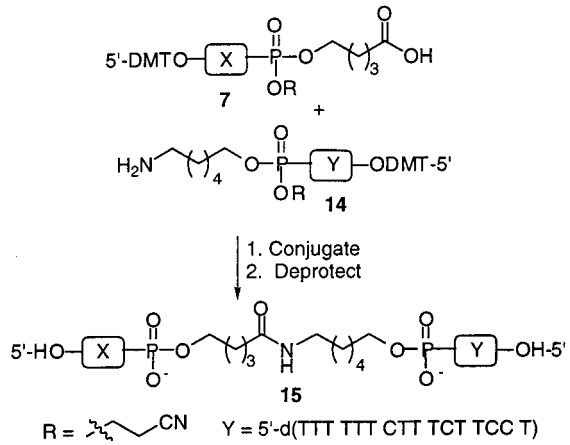
Table 1. Segmental Synthesis of Oligonucleotides Using Protected Oligonucleotides Containing 3′-Alkyl Carboxylic Acids and 5′-Alkylamines



product	X (length in nucleotides)	% yield[a]	trials
10	5′-d(TCA GT) (5)	56.7 ± 0.5 (55.5)	3
11	5′-d(TCA GTT CAG T) (10)	60.2 ± 1.1 (54.2)	3
12	5′-d(TCC TTC TTT CTT TTT T) (16)	60.5 ± 0.3 (36.1)	3
13	5′-d(TTT TTC CTT CTT TCT TTT TTT TTT) (24)	60.2 ± 3.1 (32.5)	4

[a] Isolated percent yields were determined as described in text. Values in parentheses are without scaling for oligonucleotide deletions.

Table 2. Segmental Synthesis Using Protected Oligonucleotides Containing 3′-Alkyl Carboxylic Acids and 3′-Alkylamines



product	X (length in nucleotides)	% yield[a]	trials
15	5′−d(TCC TTC TTT CTT TTT T) (16)	77.0 ± 3.3 (46)	3

[a] Isolated percent yields were determined as described in text. Values in parentheses are without scaling for oligonucleotide deletions.

acid containing components. Percent yields were determined on the basis of limiting substrate (5′-alkylamine oligonucleotide) in two ways (Table 1). First percent yields were determined by comparing the amount of isolated product compared to recovered substrate, which was subjected to all of the same manipulations. This method of determining yields has been employed in all previous reports of oligonucleotide conjugation involving protected oligonucleotides.[13−17] In addition, percent yields were adjusted to account for the fraction of carboxylic acid containing oligonucleotide that was not full length. Since all deletions in the crude photolyzate contain a 3′-carboxylic acid, the total concentration of biopolymers containing this functional group is greater than that measured via the dimethoxytrityl cation assay. Deletions were accounted for by dividing the yield determined as described above by the fraction of the carboxylic acid component that contains the full-length oligonucleotide, which in turn was based upon the dimethoxytrityl cation yields during the oligonucleotide synthesis. An assumption in this analysis is that those oligonucleotides of varying length couple equally and is supported by the data (Table 1).

After accounting for competition by deletions, isolated yields of oligonucleotides were close to 60% and were independent of the length of the carboxylic acid component (Table 1). Attempts to increase the yield of segment coupling product by changing solvents (i.e., acetonitrile/1,2-dichloroethane, 1:1), increasing the number of equivalents of carboxylic acid component to 5, or increasing the reaction time from 2 to 10 h had no effect (data not shown).

Solution-Phase Segment Coupling of Protected Oligonucleotides Containing 3′-Alkyl Carboxylic Acids with Protected Oligonucleotides Containing 3′-Alkylamines. Segmental synthesis of oligonucleotides covalently linked at their 3′-termini could be particularly useful for applications requiring parallel nucleic acid strands or those in which the directionality of a single strand needs to be reversed. Currently, the most common method for preparing such biopolymers requires "reverse phosphoramidites" in which the phosphoramidite moiety is bonded to the 5′-hydroxyl and the acid labile dimethoxytrityl group protects the 3′-secondary alcohol.[20] Such reagents are commercially available but are very expensive. The coupling method described here enables one to alter the relative polarity of the oligonucleotide segments by altering the terminus to which the electrophile or nucleophile is attached. This concept was demonstrated using the 16mer 3′-carboxylic acid containing biopolymer (**7**) employed above (Table 1) and a protected 16mer containing a 3′-alkylamine (**14**). The latter was obtained from **4** as previously described and was used as the limiting reagent.[14,21] The yield of product **15** (Table 2) was determined in two ways, as discussed above, and was found to be comparable to the respective product (**12**) obtained using 5′-alkylamine containing oligonucleotide (Table 1).

Summary

Previously developed methods for preparing oligonucleotide conjugates using protected biopolymers containing a single reactive group have been extended to coupling two oligonucleotides to one another. The method does not require any templating, and the yields are independent of the sequence or length of biopolymer substrates over the range studied (5−24 nucleotides). Conjugation is effected via a nonnative amide linkage under mild conditions in good yields. This method will

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be useful for the convergent synthesis of oligonucleotides containing nonnative linkages.[7,20,22]

Experimental Section

Oligonucleotides were synthesized on an ABI 380B DNA synthesizer using standard 1-μm cycles and β-cyanoethyl protected phosphoramidites. The exocyclic amines of deoxyadenosine, deoxycytidine, and deoxyguanosine were protected as their isobutyryl amides.[23] With the exception of solid-phase supports **1**, **2**, and **4** and the phosphoramidite for deoxycytidine (Pharmacia), all DNA synthesis reagents were obtained from Glen Research, Inc. Protected oligonucleotides were cleaved photochemically from their supports as previously described.[13,14] Benzotriazol-1-yloxytri-pyrrolidinophosphonium hexafluorophosphate (PyBOP) was obtained from Aldrich. Dimethylformamide (DMF), diisopropylethylamine (DIEA), dichloroethane, and CH_3CN were freshly distilled from CaH_2. Electrospray mass spectrometry was carried out using a Fisons VG-Quattro. Samples were precipitated twice from NH_4OAc prior to analysis.

General Procedure for Coupling of Protected Oligonucleotides. A 33 mM solution of PyBOP and DIEA in DMF was prepared in an oven-dried vial. The protected oligonucleotides, which were stored as a solution (1:1 CH_3CN/H_2O) at −80 °C, were prepared for reaction by transferring the desired amount of material to a clean reaction flask, followed by evaporative centrifugation.[14] To the dried DNA (50 nmol of 5′- or 3′-alkylamine, 100 nmol of 3′-alkyl carboxylic acid) was added DMF (42 μL), followed by 8 μL of the solution of coupling reagents. The reaction was stirred at 25 °C for 2 h and then quenched with H_2O (100 μL). The reaction mixture was evaporated to dryness and treated with concentrated aqueous ammonia (300 μL) for 6 h at 55 °C. Following evaporation, the DNA was treated with 80% acetic acid (150 μL) for 20 min. The reaction was quenched with ethanol (150 μL) and concentrated in vacuo. Denaturing gel purification of the conjugated oligonucleotide was carried out on 20% polyacrylamide. The product band was excised, crushed, and eluted with NaCl (0.2 M)/EDTA (1 mM) overnight. Following desalting (C_{18} cartridge), isolated yields were determined by comparing the amount of oligonucleotide obtained (OD_{260}) from the conjugation reaction to the amount of unconjugated material recovered from the identical deprotection, purification, and isolation conditions.

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Supporting Information Available: Electrospray mass spectra of oligonucleotide coupling products (**10**−**13**, **15**). This material is available free of charge via the Internet at http://pubs.acs.org.

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