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NUCLEOSIDES AND NUCLEOTIDES. 162. FACILE SYNTHESIS OF 5'-5'-LINKED OLIGODEOXYRIBONUCLEOTIDES WITH THE POTENTIAL FOR TRIPLE-HELIX FORMATION[1]

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Key Words: triple-helix formation; post-synthetic modification method; 5'-5'-linked oligodeoxyribonucleotides; disulfide bond

Abstract: The facile synthesis of 5'-5'-linked oligodeoxyribonucleotides (ODNs) with the potential for triple-helix formation is described. ODNs containing 5-trifluoroethoxycarbonyl-2'-deoxyuridine were treated with several diaminoalkanes to give ODNs carrying amino-linkers. After conversion of the amino-linkers in the ODNs to mercapto-linkers, two ODNs were linked by a disulfide bond to give 5'-5'-linked ODNs. The thermal stability of these ODNs with target duplexes was also studied.

Recent studies of oligonucleotide-directed triple-helix formation have been of considerable interest. Chemically synthesized oligodeoxyribonucleotides (ODNs) and their analogues have been used for biological and biochemical studies, such as site-specific cleavage of DNA and inhibition of DNA-protein binding.[2] Two major classes of triple helices can be identified based on the orientation of the third strand. When the third strand consists mainly of pyrimidines, Hoogsteen-type Py • PuPy base triplets (T • AT and C^+ • GC) are formed in which the third strand is parallel to the purine strand of the target duplex. On the other hand, when the third strand is predominantly purines, Pu • PuPy-type base triplets (G • GC and A • AT) are formed in which the third strand is antiparallel to the purine strand of the target duplex. However, the number of target sequences in such studies is limited because homopurine clusters with fairly long chain lengths are required for stable triple-helix formation. Several approaches have been developed to overcome this limitation.[3-14] These allow for the targeting of sequences made up of adjacent blocks of purines and pyrimidines by using the third strand to pair with purines on alternate strands by cross-overs in the major groove. This has been achieved by two strategies. One strategy[3-7] uses a natural ODN and two classes of base triplets, Py • PuPy and Pu • PuPy, and the other strategy[8-14] uses only one set of base triplets, Py • PuPy and a modified ODN containing an internal 3'-3' or 5'-5' linkage. In the latter case, only 3'-3'-linked ODNs have given nearly satisfactory results. It is difficult to design 5'-5'-linked ODNs because the distance between the 5'-ends of the third strands in the triple helices is much longer than that between the 3'-ends.[9] Therefore, a linker group with a suitable length is needed for stable triple-helix formation.

Recently, we developed a new and convenient method for the post-synthetic modification of ODNs with various amino-linkers using 5-methoxycarbonyl-2'-deoxyuridine (**MCdU**) as a convertible nucleoside (**CN**).[15] By treating ODNs containing **MCdUs** at various positions with several diaminoalkanes, the desired ODNs containing 5-(*N*-aminoalkyl)carbamoyl-2'-deoxyuridines (**ACdUs**) can be readily obtained. Using this method, we can determine the optimum length of the linker for a desired function without separately synthesizing several mononucleoside units with various linker lengths. From these findings, we envisioned

d-$^{3'}$TTTMMTMMU$^{5'}$-(L2)-$^{5'}$UMMTMMTTT$^{3'}$ I

d-$^{3'}$TTTMMTMMU$^{5'}$-(L3)-$^{5'}$UMMTMMTTT$^{3'}$ II

d-$^{3'}$TTTMMTMMU$^{5'}$-(L4)-$^{5'}$UMMTMMTTT$^{3'}$ III

d-$^{3'}$TTTMMTMMU$^{5'}$-(L7)-$^{5'}$UMMTMMTTT$^{3'}$ IV

L2 = -CONH$(CH_2)_2$NHCO$(CH_2)_2$S-S$(CH_2)_2$CONH$(CH_2)_2$NHCO-

L3 = -CONH$(CH_2)_3$NHCO$(CH_2)_2$S-S$(CH_2)_2$CONH$(CH_2)_3$NHCO-

L4 = -CONH$(CH_2)_4$NHCO$(CH_2)_2$S-S$(CH_2)_2$CONH$(CH_2)_4$NHCO-

L7 = -CONH$(CH_2)_7$NHCO$(CH_2)_2$S-S$(CH_2)_2$CONH$(CH_2)_7$NHCO-

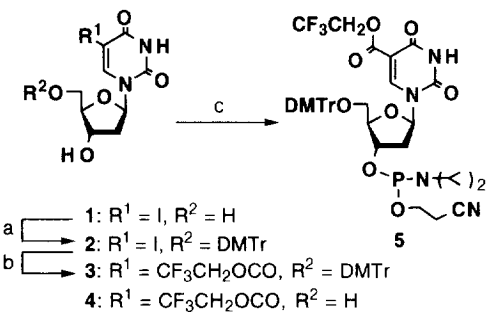
Figure 1. List of 5'-5'-linked ODNs synthesized. T and M correspond to thymidine and 5-methyl-2'-deoxycytidine, respectively.

that if we could convert the amino-linkers in the ODNs to mercapto-linkers and link the two ODNs by a disulfide bond, we could readily determine the optimum length of the linker group for stable triple-helix formation.

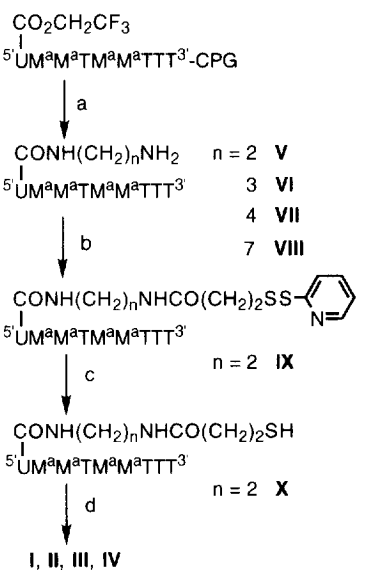
In this communication, we report the synthesis of symmetrical 5'-5'-linked ODNs linked at the 5-position of 2'-deoxyuridine residues (Figure 1). The thermal stability of these ODNs with a target duplex and a gel retardation assay were also studied.

Synthesis. In a previous paper,[15] we used **MCdU** as a **CN**. However, to ease the conversion of the **CN** to the desired nucleoside in the ODN, we chose 5-trifluoroethoxycarbonyl-2'-deoxyuridine (**4**) as a new **CN**. The synthesis of phosphoramidite unit **5** is outlined in Scheme 1. Palladium-catalyzed carbonylation of 5'-*O*-dimethoxytrityl-5-iodo-2'-deoxyuridine (**2**) with carbon monoxide in CF_3CH_2OH gave **3** in 82% yield,[16] which was phosphitylated to give phosphoramidite unit **5** in 74% yield.[17]

Compound **5** was incorporated into 9-mer [5'-**4**M^{a}M^{a}TM^{a}M^{a}TTT-3'-CPG, where T is thymidine, M^a is N^4-acetyl-5-methyl-2'-deoxycytidine, and CPG is a controlled-pore glass] using the phosphoramidite method on a DNA synthesizer. The fully protected ODN (1 μmol) linked to a solid support was treated with a large excess of diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, or 1,7-diaminoheptane in MeOH at 55 °C overnight, followed by C-18 column chromatography, and de-tritylation gave ODN **V**, **VI**, **VII**, or **VIII** in 20-32 OD_{260} units (Scheme 2).



Scheme 1. a) DMTrCl, pyridine, r.t. 99% (**2**); b) CO, $(PhCN)_2PdCl_2$, CF_3CH_2OH, Et_3N, CH_3CN, 60 °C. 82% (**3**); c) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, *N,N*-diisopropylethylamine, CH_2Cl_2, r.t. 74% (**5**).



Scheme 2. a) $H_2N(CH_2)_nNH_2$, MeOH, 55 °C; b) *N*-succinimidyl-3-(2-pyridyldithio) propionate; c) DTT; d) O_2. T, M, and M^{a}correspond to thymidine, 5-methyl-2'-deoxycytidine, and N^4-acetyl-5-methyl-2'-deoxycytidine, respectively.

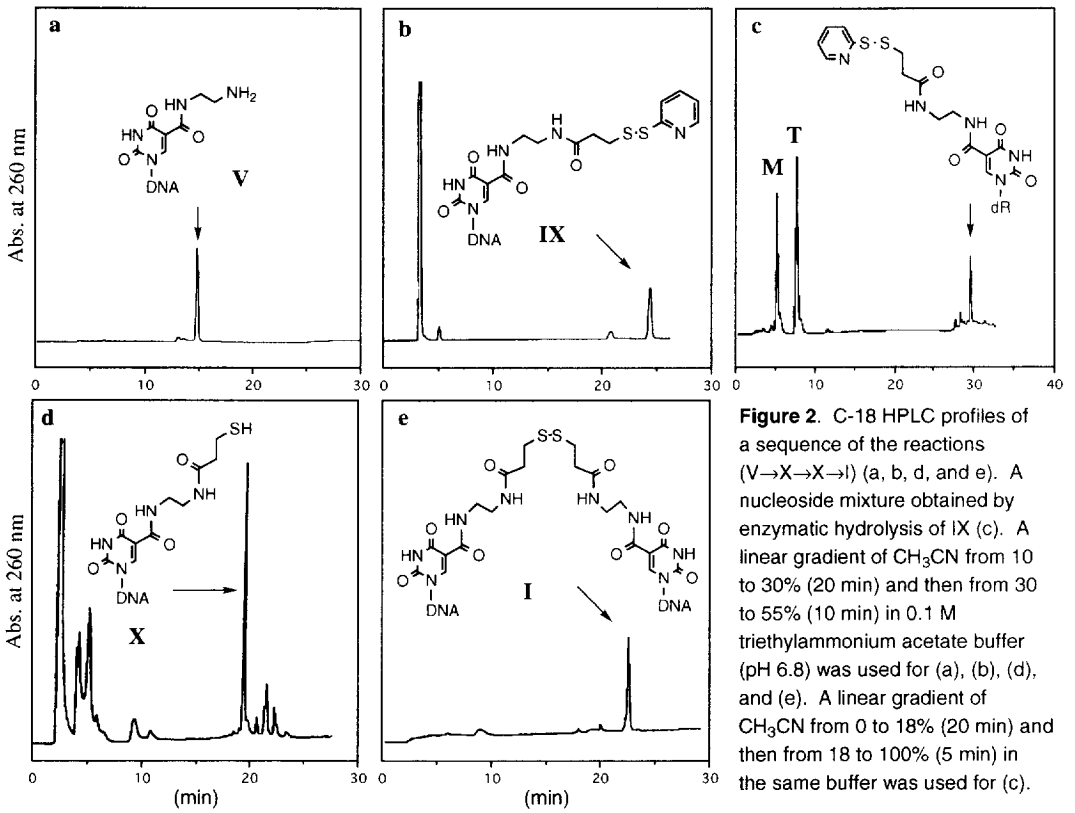


Figure 2. C-18 HPLC profiles of a sequence of the reactions (V→X→X→I) (a, b, d, and e). A nucleoside mixture obtained by enzymatic hydrolysis of IX (c). A linear gradient of CH_3CN from 10 to 30% (20 min) and then from 30 to 55% (10 min) in 0.1 M triethylammonium acetate buffer (pH 6.8) was used for (a), (b), (d), and (e). A linear gradient of CH_3CN from 0 to 18% (20 min) and then from 18 to 100% (5 min) in the same buffer was used for (c).

The synthesis of the 5'-5'-linked ODNs is outlined in Scheme 2. Typical C-18 HPLC profiles of the reactions are shown in Figure 2. First, ODN **V** (4.5 OD_{260} units) was treated with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) in a 50 mM $NaHCO_3$-Na_2CO_3 buffer (pH 10.8) at room temperature for 30 min to give ODN **IX** (2.5 OD_{260} units) (Figure 2b), the structure of which was confirmed by enzymatic digestion with snake venom phosphodiesterase and calf intestine alkaline phosphatase, as shown in Figure 2c. Next, ODN **IX** (1.25 OD_{260} units) was treated with 6 mM dithiothreitol (DTT) to give ODN **X**, and the reaction was analyzed by C-18 HPLC (Figure 2d). The major product (**X**) was collected and oxidized under an O_2 atmosphere. Analysis of the reaction mixture by C-18 HPLC after 12 h showed the formation of a slower-eluting product (**I**) (Figure 2e). ODN **I** was obtained in 0.35 OD_{260} units, and its structure was supported by the fact that exposure of the product to DTT led to the formation of the thiolated compound ODN **X** (data not shown). ODNs **II**, **III**, and **IV** were obtained in a similar mammer.

Studies of Triple-helix Formation by Thermal Denaturation. To study the effect of these linker lengths on triple-helix formation, we examined the stability of triple helices formed by these ODNs with duplexes **1** and **2** by thermal denaturation in a buffer at pH 6.0. The sequences of duplexes **1** and **2** are shown in Figure 3. Duplex **1** contains two separated polypurine binding domains, one on each strand of the duplex. The sequences of the binding domains are symmetrical. On the other hand, duplex **2** contains one extra base pair

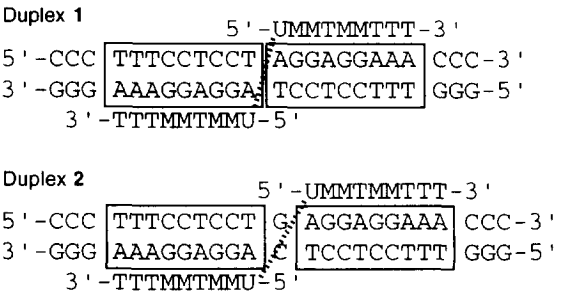


Figure 3. The sequences of the target duplexes.

(G·C) between two binding domains, which does not participate in Hoogsteen base-pair interactions with the third strand. This base pair is spanned by the linker groups. Two equivalents of unlinked ODNs (**V-VIII**) and one equivalent of 5'-5'-linked ODNs (**I-IV**) to duplexes **1** and **2** were used for the thermal denaturation. When duplex **1** was used as a target DNA, two transitions were observed in the melting profiles of all the triple helices at pH 6.0: transitions with higher Tms due to melting of target duplex **1** (73 °C) and transitions with lower Tms corresponding to dissociation of the third strands from the triple helices. As shown in Figure 4a, the Tms of dissociation of the unlinked ODNs (**V-VIII**) were around 17 °C. On the other hand, the Tms of the 5'-5'-linked ODNs (**I-IV**) (about 30 °C) were significantly higher than those of unlinked ODNs. The stabilities of triple helices formed by the 5'-5'-linked ODNs and duplex **1** depended on the length of the alkyl-linkers. ODN **I**, which bore the shortest linker group, formed the most stable triple helix in the series. With duplex **2** as a target DNA, similar melting profiles were observed. As shown in Figure 4b, the Tms of dissociation of the 5'-5'-linked ODNs with duplex **2** were slightly lower than those of the 5'-5'-linked ODNs with duplex **1**, but significantly higher than those of the unlinked ODNs with duplex **1**. Furthermore, ODN **I**, which bore the shortest linker group, formed the most stable triple-helix.

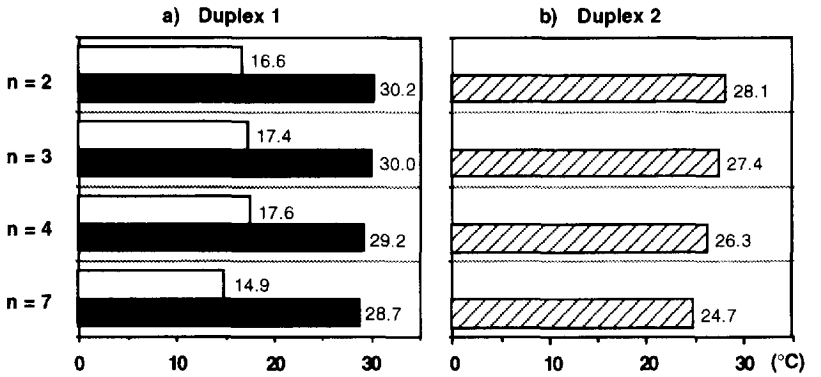


Figure 4.[18] a) Tms (°C) of the triple helices formed by the ODNs and duplex **1**. b) Tms (°C) of the triple helices formed by the ODNs and duplex **2**: open bars, duplex **1** - ODN **V**, **VI**, **VII**, or **VIII**; filled bars, duplex **1** - ODN **I**, **II**, **III**, or **IV**; hatched bars, duplex **2** - ODN **I**, **II**, **III**, or **IV**.

Gel Retardation Assay. The formation and stability of triple helices were also confirmed by a gel retardation assay.[19] After one strand of duplex **1** was labeled with ^{32}P, 10 pmol of 5'-5'-linked ODN **I** or 20 pmol of unlinked ODN **V** was added to 20 pmol of target duplex **1**, and the samples were analyzed by a non-denaturing 20% polyacrylamide gel electrophoresis containing 100 mM NaOAc (pH 5.0), 50 mM NaCl, and

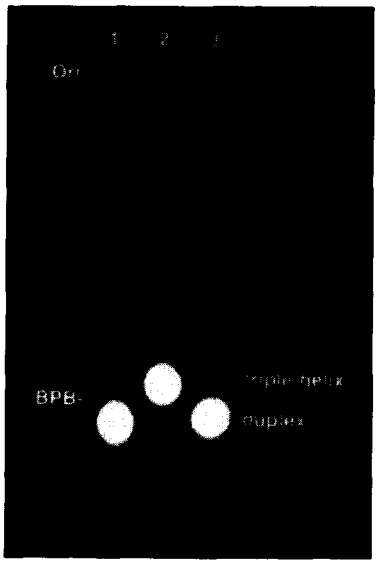


Figure 5.[19] 20% Non-denaturing polyacrylamide gel electrophoresis (pH 5.5) at 4 °C: lane 1, duplex **1**; lane 2, duplex **1** - ODN I; lane 3, duplex **1** - ODN **V**.

100 mM $MgCl_2$. As shown in Figure 5, the 5'-5'-linked ODN (lane 2) converted almost all of the duplex to triple-helix species, while unlinked ODN **V** (lane 3) failed to convert a significant amount of duplex to triple-helix. These results indicate that 5'-5'-linked ODNs form more stable triple helices than unlinked ODNs under these conditions.

Conclusion. In this paper, we reported a novel and convenient method for synthesizing 5'-5'-linked ODNs with the potential for triple-helix formation. Among the ODNs synthesized here, ODN **I**, which bore the shortest linker group, formed the most stable triple helix with either duplex **1** or **2**. In this study, we used a symmetrical sequence to simplify the synthesis of the 5'-5'-linked ODNs. However, by using double-stranded DNA as a template, 5'-5'-linked ODNs with asymmetrical sequences can be readily synthesized on the duplex. These results will be reported shortly.

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16. Synthesis of **3**: A mixture of **2** (4.22 g, 6.43 mmol), bis(benzonitrile)palladium dichloride (50 mg, 0.129 mmol), Et_3N (2.12 ml, 12.9 mmol), and CF_3CH_2OH (4.61 ml, 64.3 mmol) in CH_3CN (50 ml) was

heated at 60 °C for 11 h under a CO atmosphere. The reaction mixture was filtered through a Celite pad and washed with EtOH. The combined filtrate and washings were concentrated, and then the residue was dissolved in $CHCl_3$. The solution was washed with aqueous saturated $NaHCO_3$ (x 2) and then brine, dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column (3.0 x 13 cm) with 0-6% MeOH in $CHCl_3$ to give **3** (3.60 g, 85% as a yellow foam): FAB-MS *m/z* 656 (M^+); ^{1}H-NMR ($CDCl_3$) δ 8.61 (s, 1H, H-6), 7.40-6.81 (m, 13H, DMTr), 6.16 (t, 1H, H-1', $J_{1',2'a} = J_{1',2'b} = 6.2$ Hz), 4.35 (q, 2H, CF_3CH_2, $J_{FH} = 8.5$ Hz), 4.18 (ddd, 1H, H-3', $J_{3',4'} = 4.0$, $J_{3',2'a} = J_{3',2'b} = 4.4$ Hz), 4.05 (q, 1H, H-4', $J_{4',3'} = J_{4',5'a} = J_{4',5'b} = 4.0$ Hz), 3.79 (s, 6H, CH_3O), 3.49-3.43 (m, 2H, H-5'a,b), 2.55, 2.26 (each ddd, each 1H, H-2'a,b, $J_{2'a,1'} = J_{2'b,1'} = 6.2$, $J_{2'a,3'} = J_{2'b,3'} = 4.4$, $J_{2'a,2'b} = 13.3$ Hz); FAB exact MS calcd for $C_{33}H_{31}F_3N_2O_9$ 656.1981, found 656.1979.

17. Synthesis of **5**: After successive coevaporation with pyridine, **3** (584 mg, 0.889 mmol) was dissolved in CH_2Cl_2 (10 ml) containing *N,N*-diisopropylethylamine (0.23 ml, 1.33 mmol). 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.297 ml, 1.33 mmol) was added to the solution and the reaction mixture was stirred for 20 min at room temperature. Aqueous saturated $NaHCO_3$ and $CHCl_3$ were added to the mixture, and the separated organic layer was washed with aqueous saturated $NaHCO_3$ (x 3), dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column (2.9 x 7 cm) with 66% EtOAc in hexane to give **5** (502 mg, 66% as a white foam): FAB-MS *m/z* 656 (M^+); ^{31}P-NMR ($CDCl_3$) δ 150.05, 149.57 (85% H_3PO_4 as an internal standard); FAB exact MS calcd for $C_{42}H_{48}F_3N_4O_{10}P$ 856.3060, found 857.3140.

18. A solution containing duplex **1** or **2** was heated at 90 °C for 10 min, and then gradually cooled to room temperature. The solution was mixed with the ODNs, and then cooled to 4 °C and used for the thermal denaturation study. Thermally induced transitions of each mixture of ODNs were monitored at 260 nm on a Perkin Elmer Lambda2S. Sample temperature was increased one degree per min. Each sample contained ODN **I**, **II**, **III**, or **IV** (3 μmol) and target duplex **1** or **2** (3 μmol) in a buffer of 0.01 M NaH_2PO_4-Na_2HPO_4 (pH 6.0) containing 0.5 M NaCl and 20 mM $MgCl_2$, or ODN **V**, **VI**, **VII**, or **VIII** (6 μmol) and target duplex **1** (3 μmol) in the same buffer.

19. For gel retardation assays of the triple helices, a non-denaturing 20% polyacrylamide gel (99 : 1 acrylamide/bis-acrylamide) containing 100 mM NaOAc (pH 5.0), 50 mM NaCl and 100 mM $MgCl_2$ was prepared and run at 4 °C using the same buffer. One strand of the target duplex **1** was labeled at the 5'-end by T4 polynucleotide kinase using γ-^{32}P-ATP.[20] Third-strand ODN **I** (10 pmol) or third-strand ODN **V** (20 pmol) was added to the target duplex **1** (10 pmol). The mixture was heated at 50 °C for 10 min and then slowly cooled to 4 °C. Samples were then loaded in 15% Ficoll (Pharmacia type 400), and bromophenol blue dye was used as a maker.

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