Synthesis and Triple Helix Formation by Alternate Strand Recognition of Oligonucleotides Containing 3′-3′ Phosphodiester Bonds

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ODNs containing a 3′-3′ phosphodiester junction have been conveniently synthesized through a solid phase procedure involving only 3′-phosphoramidite nucleosides, starting from a modified support linking the first nucleotide through the base. Thermal denaturation studies showed that 16-mers with this inversion of polarity cooperatively bound to 5′-(purine)$_m$(pyrimidine)$_n$-3′-type duplexes by specific recognition of the oligopurine domains alternately on the two DNA strands. The nature of the 3′-3′ junction and nearest neighbors affected the stability of the resulting triplexes.

Introduction

The "antigene" approach is based on sequence specific recognition of a DNA segment by a single-stranded, synthetic oligodeoxyribonucleotide (ODN) annealing in the major groove of the target duplex to give a triple helical complex.[1] While extremely fascinating in principle for its high potential in the control of gene expression at both transcriptional and replicational levels, this strategy has still limited possibilities of pharmaceutical and biological applications. One of the major limitations is that a stable triple helix, under physiological conditions, either via Hoogsteen or reverse-Hoogsteen triads formation, can be envisaged only for relatively long (15−17 bases) homopurine tracts within the same strand of a double helical DNA fragment: such a requirement is not always met in biologically important targets (tracts involved in gene control, protein binding, etc.).

To recognize a wider number of relevant DNA sequences, several approaches have recently been proposed. Some new base analogues, ad hoc designed to selectively bind to the pyrimidines in T−A or C−G Watson−Crick base pairs, have been reported to form stable triads under physiological conditions.[2−6] On the other hand, many research groups[7−16] have explored another approach, aimed at targeting (purine)$_m$(pyrimidine)$_n$ sequences with the third strand hybridizing the adjacent purine blocks on alternate strands, by switching strand at the junction between the oligopurine and oligopyrimidine domains. Within the best-studied triplex motif Py·Pu−Py (where a polypyrimidine runs parallel to the homopurine domain of the target duplex forming Hoogsteen triplets), a simultaneous binding to adjacent purine tracts on alternate strands of the duplex can be achieved provided that two conditions are fulfilled:

(1) the ODN must be able to efficiently cross the major groove, without causing heavy distortions to the triplex conformation;

(2) it must have a convenient linker introducing the appropriate inversion of polarity, i.e. a 3′-3′ or 5′-5′ internucleoside junction, which ensures for both the linked 5′- or 3′-ends the required orientation.

Several linkers, imposing the desired inversion of polarity as well as ensuring a high degree of cooperativity to the linked domains, have been described to covalently connect the two 3′- or 5′-termini of the triple helix forming ODN. Among others, 1,2-dideoxy-D-ribose,[7] 1,3-propanediol[7] or its oligomers,[8] an *o*-xylenyl unit[9,10] and an interbase linkage[14−16] were reported to efficiently tether 3′-ends in modified ODNs which gave stable triplexes. Less interesting proved to be 5′-5′ tethered ODNs for alternate strand recognition; in all the studied cases, affinity toward the target DNA dramatically diminished when such a strand switch was introduced.[8] However the already reported syntheses of ODNs having either a 3′-3′ or a 5′-5′ junction suffer from two common drawbacks:

(1) preparation of the dimeric building blocks containing the appropriate 3′-3′ or 5′-5′ linkage needs several and sometimes troublesome solution steps;

(2) the use of both the 3′- and the expensive 5′-phosphoramidites is required.

In this paper we report a convenient solid-phase synthesis of ODNs containing a 3′-3′ internucleoside phosphodiester linkage, on a modified support linking the first nucleotide through the base and employing uniquely 3′-phosphoramidite nucleosides for the ODN assembly.

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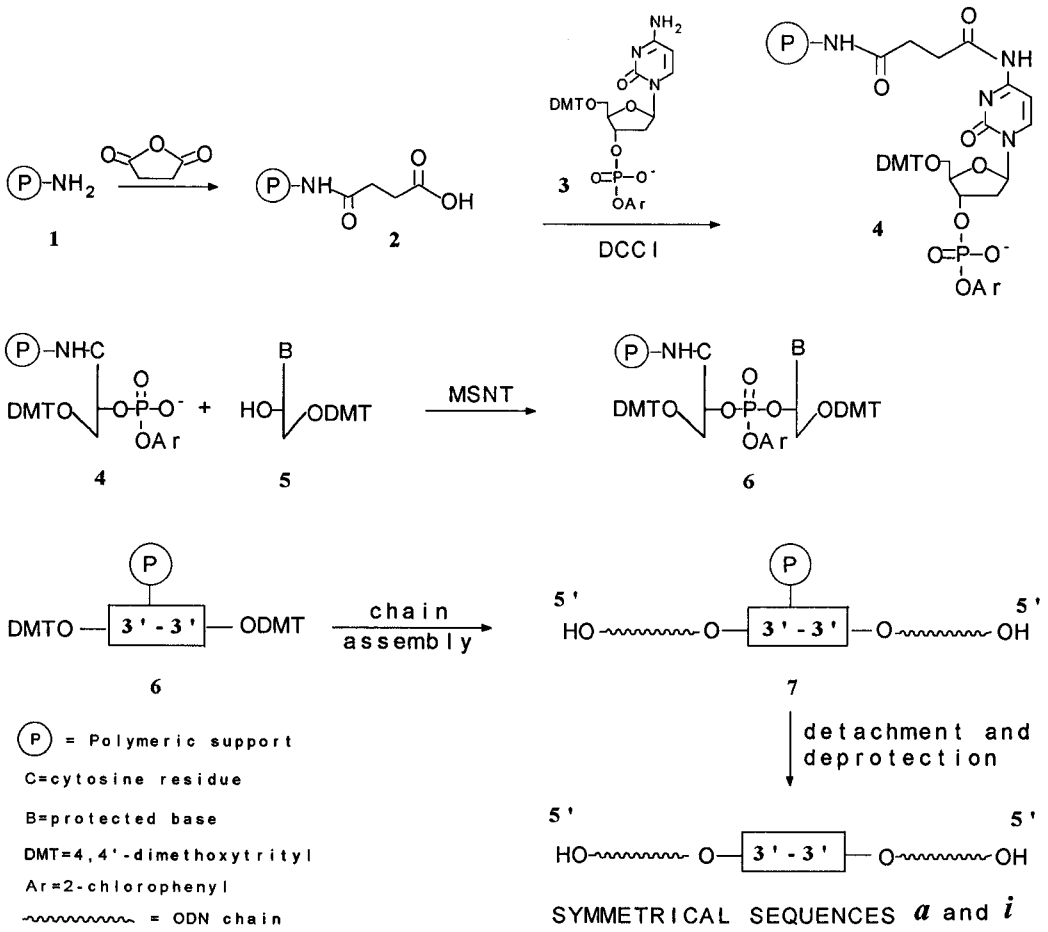
Scheme 1



Thermal denaturation analysis and gel retardation assays provided evidence that these oligomers were able to form intermolecular triplex complexes by alternate strand recognition; the different stabilities of selected 16-mers have been analyzed as a function of the nature of the 3′-3′ linked nucleosides and of the flanking bases.

Results and Discussion

Derivatization of the Solid Support. Synthesis of Symmetrical and Asymmetrical Sequences. The synthetic approach chosen for the preparation of oligomers with a 3′-3′ phosphodiester internucleosidic (3′-p-3′) bond is based on the incorporation as the first unit on the solid support of a nucleoside-3′-phosphodiester through the base exocyclic amino function. The resulting functionalized support resulted to be particularly useful to our purposes, allowing us to use the 3′-phosphodiester moiety to create the 3′-p-3′ internucleosidic bridge, while the 5′-OH group was exploited for the classical chain elongation. According to a procedure previously described for the solid-phase synthesis of cyclic ODNs,[17−20] Tentagel resin (**1**, Scheme 1), a polystyrene−polyethylene glycol copolymer of widespread use in solid-phase synthesis of peptides[21] and oligonucleotides,[22] was first treated with succinic anhydride, yielding **2**, and successively reacted with 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-deoxycytidine-3′-*O*-(2-chlorophenyl phosphate) (**3**)[20] in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCCI). In this work **3** was attached to the support with a loading of 0.10−0.12 mequiv/g; following a slightly modified procedure,[18] the other nucleosides can be anchored to the polymeric matrix through the base and, therefore, be inserted in the 3′-3′ junction.

Two reaction routes were then applied, depending if symmetrical or asymmetrical sequences with respect to the dinucleotide box linked through the 3′-p-3′ internucleosidic bond had to be prepared. In the first case (Scheme 1), it used previously synthesized support **6**, by reaction of **4** with the 5′-DMT-2′-deoxynucleoside (**5**, B = thymine, T or cytosine, C) using MSNT as activator of the 3′-(2-chlorophenyl)phosphate moiety, following a classical phosphotriester procedure.[23] Coupling yields were found to be usually slightly superior with 5′-DMT-thymidine than with 5′-DMT-2′-deoxycytidine, and typically in the range 80−87%, as calculated spectrophotometrically on the basis of the increment of the DMT cation released by acidic treatment on weighed amounts of support **6**. To check the correct functionalization, supports **6**, with B = T or C, were deprotected at the 5′-ends and then treated with concentrated ammonia, thus releasing C-3′-p-3′-C and C-3′-p-3′-T dimers, whose structures were confirmed by comparison (^{1}H NMR and

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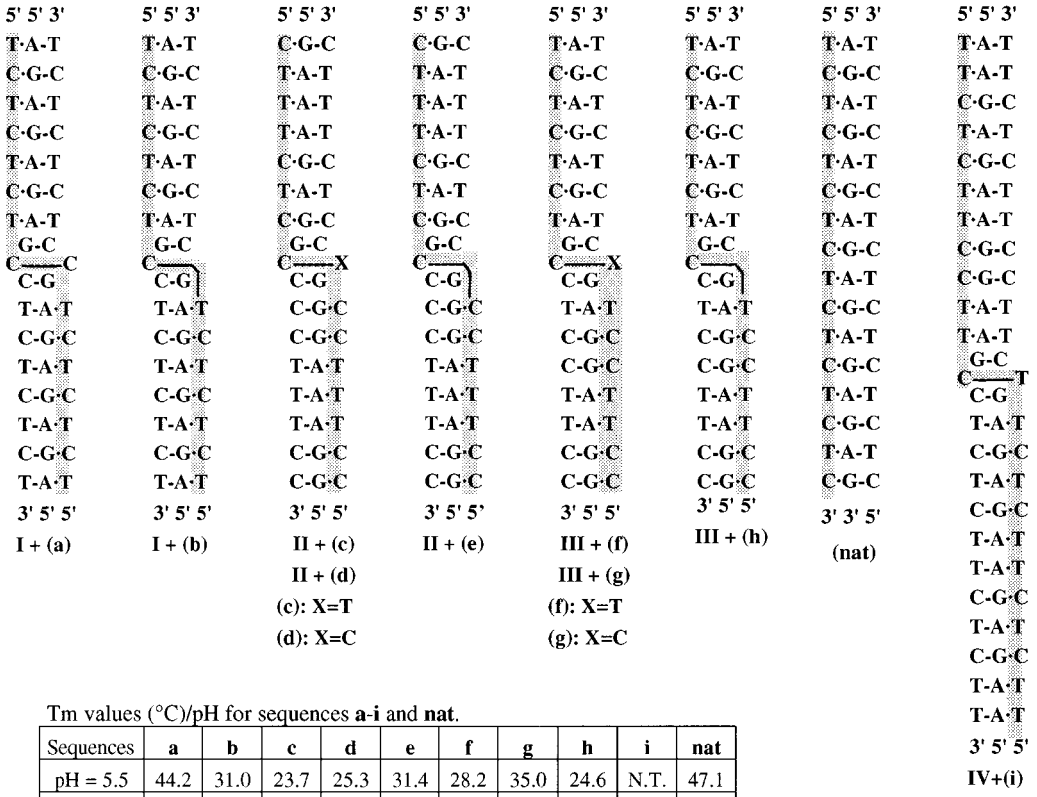
Table 1

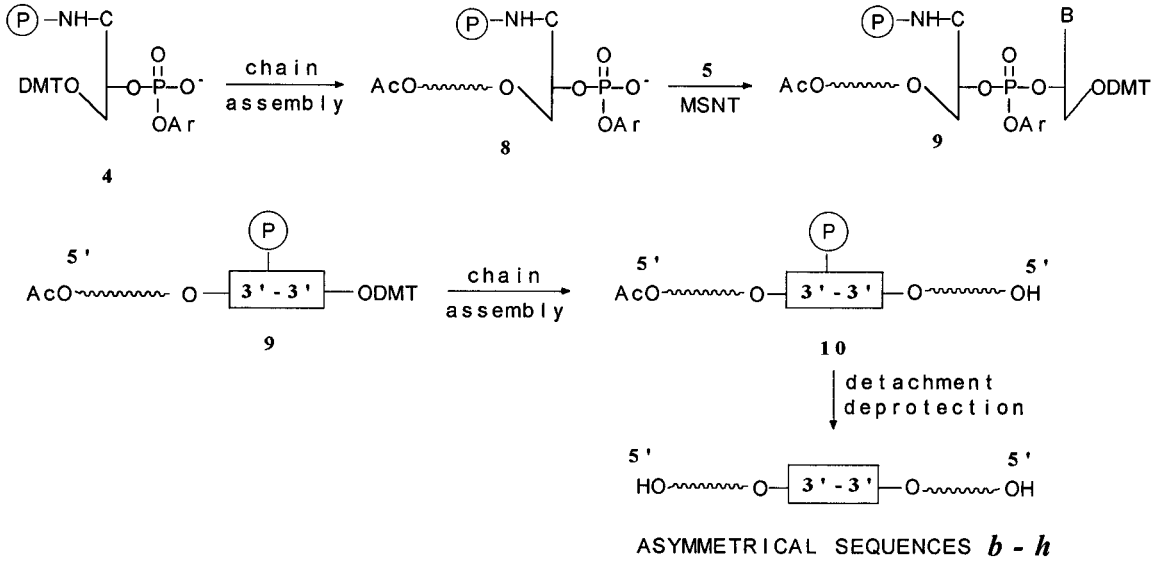


Tm values (°C)/pH for sequences **a**-**i** and **nat**.

Sequences	a	b	c	d	e	f	g	h	i	nat
pH = 5.5	44.2	31.0	23.7	25.3	31.4	28.2	35.0	24.6	N.T.	47.1
pH = 6.0	36.8	22.9	18.6	19.1	19.5	21.2	24.2	19.0	40.1	39.3
pH = 6.6	19.8	16.7	N.D.	N.D.	N.D.	19.4	19.6	17.3	27.2	24.2

N.T. = not tested; N.D. = not detected

Scheme 2



HPLC) with authentical samples obtained following standard solution methods (see Experimental Section). Chain elongation was then carried out on support **6** on a 5 μmol scale using 45 mg/mL of 3′-phosphoramidite building blocks, obtaining **7**. It is to be noted that the chain assembly on both the 5′-ends could be done simultaneously, reducing by half the number of necessary coupling steps. For oligomers **a** and **i** (see Table 1), coupling efficiency, checked by spectroscopic measurements of the DMT cation released by the DCA treatments, was found always superior to 96%. Moreover in the case of 16-mer **a** the correct growing of both the 5′-ends of the chain was monitored by analyzing ion exchange HPLC profiles of the detached material from samples of the resin taken after each coupling cycle.

In the reaction route developed for the synthesis of asymmetrical sequences **b**−**h** (Scheme 2), the elongation on the 5′-end of support **4** was first performed, producing the first half of the ODN chain. Then the final 5′-DMT group was removed and the resulting 5′-hydroxy function acetylated, giving **8**. As previously proved,[20,24] the presence of a 3′-phosphodiester moiety on support **4** did not cause interferences with the chain elongation via phosphoramidite chemistry; support **8** was successively re-

acted with the 5′-DMT-2′-deoxynucleoside **5** (B = C or T) in the presence of MSNT. Incorporation yields of **5**, creating the 3′-3′ junction in **9**, were measured as previously described by quantitative DMT cation tests and were comparable to those obtained in the procedure above described for symmetrical sequences. The second domain of the desired oligomer could then be assembled following standard phosphoramidite protocols.[25]

After detachment from supports **7** or **10** and removal of the base and phosphate protecting groups by concentrated ammonia treatment, crude oligomers **a**−**i** were purified by HPLC on a DEAE ion exchange column, using a linear gradient of KCl in a 20 mM K_2HPO_4 aqueous solution containing 20% (v/v) CH_3CN and successively desalted on a Biogel P2 column. Purity of the isolated products was checked by reverse phase HPLC analysis. In representative cases, analysis by MALDI-TOF mass spectrometry was performed, giving masses in accordance with the expected structures.

Thermal Denaturation Studies. Triple helix formation of oligomers **a**−**i** with the corresponding DNA target duplexes (**I**, **II**, **III**, **IV**) was monitored by recording melting and hybridization curves at 260 nm, which at the applied temperature gradient (0.5 °C/min) were always superimposable, thus demonstrating equilibrium binding processes. Since all the selected sequences contained cytosines, which in the Hoogsteen pairing mode have to be protonated in order to recognize a GC couple, pH effects were taken into consideration and different conditions were tested (typically pH 5.5, 6.0, 6.6 and 7.2). The T_m values relative to triplex−duplex transitions are summarized in Table 1. As an example, the melting curves of the triplexes formed by oligomers **a** and **b**, respectively, with the target duplex **I** at pH = 6.0, carried out in 5 mM $MgCl_2$, 140 mM KCl, 5 mM NaH_2PO_4 (1 μM each strand, approximately) are reported in Figures 1a,b.

Thermal denaturation experiments at acidic pH showed, in all cases, two well defined and separated S-shaped transitions; those at the highest temperatures resulted to be independent from pH conditions and from the concentration of the 3′-3′ linked oligomer, and were therefore attributed to the melting of the target duplexes into random coils. Conversely, the transitions at the lowest temperatures in all cases appeared at decreasing T values with increasing pH (when operating at pH = 7.2 no such transition could be evidenced over 10 °C) and showed lower hyperchromicities when the ratio 3′-3′ linked oligomer/duplex was inferior to 1:1; these were so ascribed to the dissociation of the 3′-3′ linked single strands from the target duplexes. It could be excluded that such a behavior was due to self-associations of the single stranded ODNs since no transition was found when heating the 3′-3′ linked oligomers in the absence of the target duplex DNA; on the other hand, melting experiments of the duplexes alone were always monophasic. Also dissociation of the third strand only partially able to pair with the duplex, with one domain of the chain dangling at the 3′-3′ junction, was proved not to be responsible for the observed processes. In fact 7-mer 5′-TCTCTCT-3′ and 8-mer 5′-TCTCTCTC-3′, when mixed with 5′-AGAGAGAGCTCTCTCT-3′ in duplex form either

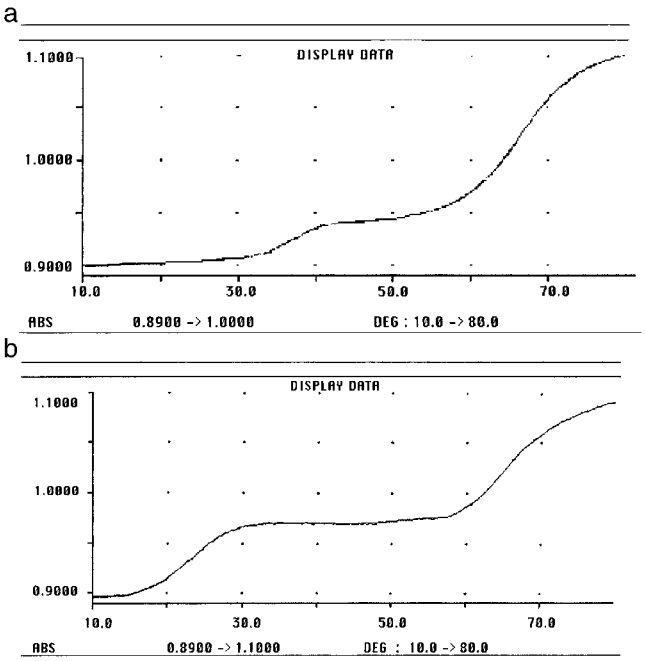


Figure 1. (a) Melting profile of oligomer **a** with the target duplex **I** at pH = 6; buffer: 5 mM $MgCl_2$, 140 mM KCl, 5 mM NaH_2PO_4; $c \sim 1$ mM each strand; 1.0 °C/min. (b) Melting profile of oligomer **b** with the target duplex **I** at pH = 6.

in 1:1 or 1:2 ratio (and, analogously, 11-mer 5′-TTCTCT-TCCTT-3′ with the 24-mer double stranded 5′-AA-GAGAAGGAAGCTCTCTTCTCTT-3′) gave melting profiles reflecting notably reduced stabilities (T_m < 10 °C at pH = 6.0) for the resulting triplex type complexes. This result confirmed a total and cooperative binding of the two 3′-3′ linked domains of the third strand in the triplex formation process.

Comparing the T_m values for all the studied cases it turned out that triple helix stability was basically affected by sequence composition effects, for the same conditions of pH, salt, T, and length of the ODN, as in the case of triplexes without a strand switch.[26,27] The highest affinity for the target was observed for symmetrical sequences having alternating cytosines and thymines; interestingly, 16-mer **a** exhibited melting temperatures not so dramatically inferior than those of a polypyrimidine ODN of the same length forming a classical triplex structure (**nat**). It can be concluded, therefore, that switching from one block of purines to the adjacent one, on the other strand of the target duplex, does not produce a sensible destabilization in the triple helical complex, hence that this type of third strand is sufficiently flexible and able to bind efficiently to both sides of the duplex. Similar conclusions can be drawn when considering 24-mer **i**. On the other hand, asymmetrical 16-mers **c**, **d**, **f**, and **g**, designed to have approximately the same ratio of C/T as in the symmetrical strand **a**, showed markedly reduced values in T_m, indicating that less stable complexes were formed. These results can be attributed to the presence of adjacent cytosines within the oligonucleotide sequence, as a result of the repulsive electrostatic forces generated by consecutive C^+GC triplets.

Apart from interactions due to cytosines within each 5′-3′ domain of the chain, the role of the 3′-3′ linked

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nucleosides, as a function of their nature, nearest neighbors, and length of the single strand, was then investigated to assess if any specific 3′-3′ "bridge" effect contributed to the overall triplex stability. To determine whether the two bases connected through the 3′-3′ phosphodiester bridge were effectively involved in some kind of recognition or acted merely as spacers connecting the two 3′-termini of the ODN, the melting behavior of some 3′-3′ linked single strands differing only for point modifications was analyzed. The relative efficiency of C-3′-p-3′-C vs C-3′-p-3′-T dimers inserted in the same sequences was evaluated in recognizing a GC/CG box of the duplex. Superimposing the melting curves of **c** with **d** and of **f** with **g**, a slight, but observable, increment in T_m values when a C was replacing a T at the inversion site could always be detected. This result argued for a not total disruption of the triplex structure at the 3′-3′ junction and was further confirmed by other experiments.

Nearest neighbor effects by bases flanking the 3′-3′ bridge were also investigated: comparing the T_m values of **c** and **d** vs those of **f** and **g**, where all the 16-mers had the same number of couples of contiguous C for each 3′-5′ domain of the oligomer, it was found that the most stable triplexes were formed by **f** and **g**, where T was the neighbor base. This could only be explained in terms of reduced number of adjacent protonated cytosines, which is an indirect evidence of the participation of the cytosines in the 3′-3′ bridge in some triplet recognition mode: in fact an isolated 2′-deoxycytidine is reported to have a pK_a of 4.3,[28] while in a structured environment as the Hoogsteen triplet its basicity is sensibly enhanced,[29,30] thus allowing a high percentage of the protonated form also at pH values closer to neutrality.

Next, the length of such 3′-3′ bridge was taken into consideration. 15-mers **b**, **e**, and **h**, in which, with respect to 16-mers **a**, **c** (**d**), and **f** (**g**), one nucleoside at the 3′-3′ junction had been eliminated, were synthesized and tested for binding with duplexes **I**, **II**, and **III**, respectively. Thermal analysis showed in all cases a triplex−duplex transition; a lower affinity was found for **b** and **h** toward the target duplexes, if compared, respectively, to 16-mers **a** and **f** (or **g**), thus indicating that skipping over a base pair at the 3′-3′ linked junction forced the third strand to be bent in a way detrimental to the sequence recognition process. Conversely, **e** resulted in a more stable triplex structure than **c** and **d** when hybridized to **II**. This apparently contradictory result could be rationalized assuming that the conformation at the junction is heavily influenced by the base sequence immediately surrounding the junction itself. In oligomers **c**, **d**, and **e** there is a couple of contiguous cytosines at each flank of the 3′-3′ linkage; since it is plausible that adjacent C^+GC triplets tend to increase the relative distance between their planes to reduce repulsive interactions, this could account for the 3′-ends of each 5′-3′ domain of the chain to be found spatially closer than in usual cases, so allowing a shorter third strand to connect more profitably the two 3′-5′ halves of the oligomer. Other evidences need certainly to be collected to further confirm this hypothesis.

Gel Electrophoresis Assays. The gel mobility analysis is a well-established technique to detect triplex structures;[31,32] triplexes, formed under appropriate pH and temperature conditions, show, as expected, a lower electrophoretic mobility on polyacrylamide gels compared to duplexes. Gel retardation and gel titration assays were performed to further demonstrate the formation of a triple helical complex between the 3′-3′ modified oligomers and the corresponding target duplexes.

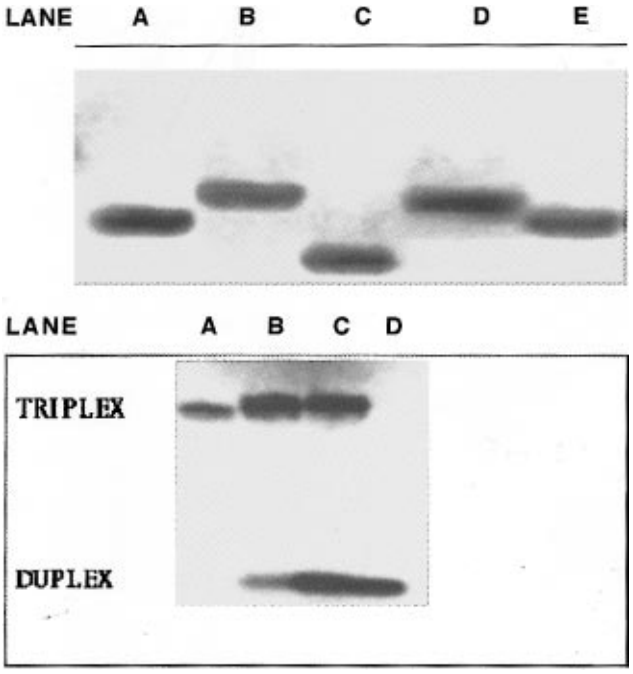

Figure 2. (a) Top: Gel mobility shift assay. Lane A: 16mer **a**; lane B: 16mer **a** + duplex **I**; lane C: duplex **I**; lane D: 15mer **b** + duplex **I**; lane E: 15mer **b**. (b) Bottom: 15% PAGE at 5 °C and pH = 6 of duplex **IV** mixed with oligomer **i** in different ratios. Lane A: duplex/oligomer 1/1; lane B: 1/0.7; lane C: 1/0.5; lane D: 1/0.

In nondenaturing polyacrylamide gels, run at pH 6.0 and 5 °C, a 1:1 mixture of the 3′-3′ oligo **a** or **b** and duplex **I** (lanes B and D, respectively, Figure 2a) yielded bands with reduced mobility compared to those given either by the single strands alone or the 16 bp duplex. Lanes A and E showed the electrophoretic behavior, respectively, of 16-mer **a** and 15-mer **b** alone, which surprisingly had a lower mobility than the duplex itself (lane C). On the other hand, when the gel was carried out under the same salt and temperature conditions, but at pH 8.0, no band attributable to a triplex structure could be visualized, while two bands, with the same mobility pattern of the 3′-3′ oligomers and the duplex, respectively, were found.

As a further evidence of the formation and the stoichiometry of the complexes in the used salt, pH, and temperature conditions, a titration experiment was performed by mixing duplex **IV** and 24-mer **i** in different ratios (Figure 2b). Judging from the intensity of the bands produced on the gel, visualized under UV rays, which correlates with the concentration of the oligomers, it could be concluded that a total complexation of the duplex was achieved with a 1:1 ratio 3′-3′ oligo/duplex, as expected for a triple helical complex.

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Conclusions

ODNs with a 3′-3′ phosphodiester bond as inversion of polarity motif were efficiently synthesized on a Tentagel resin previously functionalized with a 2′-deoxycytidine-3′-phosphodiester residue attached through the base; such a modified solid support allowed us to use only nucleosides 3′-phosphoramidite for the chain assembly. Melting curves and gel mobility shift analyses showed that these ODNs were able to bind to DNA double helical fragments of the type $(purine)_m(pyrimidine)_n$ by alternate strand recognition via Hoogsteen triplets formation. A marked sequence-dependent behavior in triplex formation was exhibited by some modified 16-mers, studied with particular attention to 3′-3′ "bridge" effects. Thermal denaturation analysis indicated that the bases connected through the 3′-3′ phosphodiester linkage assured a high degree of cooperative binding to the two 3′-5′ halves of the third strand and contributed, to some extent, to the sequence specific recognition process. As evidence in favor of such hypothesis, it was found that C-3′-p-3′-C dimer worked better than C-3′-p-3′-T in recognizing a GC/CG box of target duplexes; 15-mers usually gave lower affinities than 16-mers in targeting 16-bases long duplexes; higher T_m values were obtained with T, instead of C, as the base flanking the 3′-3′ juction.

NMR studies aimed at a deeper insight of the conformation assumed by such triplexes at the inversion site are actually underway in collaboration with another team and will be published elsewhere.

Experimental Section

Materials and Methods. Tentagel resin was purchased from Rapp Polymere, Tubingen, Germany. The solid support functionalizations were carried out in a short glass column (5 cm length, 1 cm i.d.) equipped with a sintered glass filter, a stopcock, and a cap. The oligonucleotides were assembled on a Millipore Cyclone Plus DNA synthesizer, using commercially available 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite nucleosides as building blocks. For the synthesis and the purification of the oligomers forming duplexes **I**, **II**, **III**, and **IV**, standard procedures were followed in all cases. HPLC analyses were carried out on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. UV measurements were performed on a Perkin-Elmer Lambda 7 spectrophotometer. Thermal denaturation experiments were run on a Cary 1E Varian spectrophotometer equipped with a Haake PG20 thermoprogrammer at 260 nm.

Preparation of Support 4. 1 g of support **1** (0.24 mmol of amino groups per g), mixed with 1 g (10 mmol) of succinic anhydride in 7 mL of anhydrous pyridine, was shaken for 16 h at room temperature. The resulting support **2** was filtered and exhaustively washed with pyridine, $CHCl_3$ and Et_2O and dried under reduced pressure. **2** was converted into desired support **4** by reaction with 385 mg (0.5 mmol) of **3** and 1 g (5 mmol) of DCCI in 10 mL of anhydrous pyridine for 16 h at room temperature under shaking. The final support **4**, washed with $CHCl_3$, CH_3OH and Et_2O, was dried under reduced pressure. Loadings of 0.10−0.12 mequiv/g of the 5′-DMT-2′-deoxynucleoside-3′-phosphodiester were typically obtained, as estimated by spectroscopic measurements (λ = 498 nm; ϵ = 71700 cm^{-1} M^{-1}) of the 4,4′-dimethoxytriphenylmethyl (DMT) cation released by acidic treatment (70% $HClO_4$/EtOH 3:2, v/v) on a weighed amount of the dried support.

Chain Assembly of Symmetrical Oligomers a and i. For the preparation of ODNs **a** and **i**, the following procedure was used: 50 mg (0.005 mequiv) of functionalized support **4** were left in contact with an anhydrous pyridine solution (1 mL) of **5** (0.1 mequiv) and 1-(2-mesitylensulfonyl)-3-nitro-1,2,4-triazole (MSNT, 0.2 mequiv) overnight at room temperature. After washings, the resulting support **6** was dried; DMT tests allowed to evaluate the incorporation yields of nucleoside **5**, which resulted to be typically in the range 80−82% in couplings with 5′-DMT-2′-deoxycytidine and 84−87% with 5′-DMT-thymidine. Chain elongation on both the 5′-ends of polymer **6** was then carried out using longer coupling cycles (8 min) and higher phosphoramidites concentrations (45 mg/mL) than in standard automated procedures,[25] yielding **7**.

Synthesis and Characterization of the 3′-3′ Linked C−T and C−C Dinucleoside Phosphates. 100 mg amount of support **6** (B = T) was first treated with 3% DCA in CH_2Cl_2 and then, after exhaustive washings, left in contact with 4 mL of a concentrated aqueous ammonia solution at 50 °C for 6 h. The detached material, filtered and concentrated in vacuo, was redissolved in H_2O and purified by HPLC on a Vertex Lichrosorb RP18 analytical column (250 × 4.0 mm, 5.0 μm) eluted with a linear gradient (from 0 to 30% in 30 min) of CH_3CN in 0.1 M aqueous triethylammonium bicarbonate buffer, pH = 7.0 (flow 0.8 mL/min, detection at λ = 260 nm). The peak with retention time 19 min 28 s, collected and lyophilized, was identified as a C-3′-p-3′-T dimer on the basis of its ^{1}H NMR spectrum: δ_H (D_2O, 270 MHz) 7.97 (1H, d, H-6 2′-deoxycytidine residue); 7.73 (1H, s, H-6 thymidine residue); 6.42−6.35 (2H, m, H-5 2′-deoxycytidine and H-1′ thymidine residue); 6.20 (1H, dd, H-1′ 2′-deoxycytidine residue); 4.31 (2H, m, H-4′); 3.92 (4H, m, H-5′); 2.73−2.42 (4H, m, H-2′); 1.98 (3H, s, CH_3 thymidine residue). The H-3′ signals were submerged by the residual solvent peak. The structure of the isolated compound was further confirmed by HPLC comparison with an authentical sample, synthesized following a classical solution phosphotriester protocol. Coupling 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-N^4-benzoyl-2′-deoxycytidine-3′-*O*-(2-chlorophenyl phosphate), triethylammonium salt (1.1 equiv), with 5′-*O*-(4,4′-dimethoxytriphenylmethyl)thymidine (1.0 equiv) in a pyridine solution of MSNT (3.0 equiv) allowed the fully protected C-3′-p-3′-T dimer in 90% yield, which, after purification, was then completely deprotected by treatment with a concentrated aqueous ammonia solution (6 h, 50 °C) and successively with 80% acetic acid (rt, 20 min).

Following similar procedures, starting from derivatized Tentagel support **4**, C-3′-p-3′-C dimer was synthesized, isolated, and characterized (data not shown).

Chain Assembly of Asymmetrical Oligomers b−h. The synthesis of oligos **b**−**h** was typically carried out starting from 50 mg (0.005 mequiv) of derivatized support **4**. Once assembled, the first domain of the ODN chain following standard automated methods,[25] the final DMT group was removed, and a prolonged (15 min) capping cycle was performed to block the 5′-end, thus obtaining **8**. The coupling with the 5′-DMT nucleoside **5** was then performed manually by treating polymer **8** with 0.1 mequiv of 5′-DMT-thymidine or 5′-DMT 2′-deoxycytidine and 0.2 mequiv of MSNT dissolved in 1 mL of anhydrous pyridine overnight at room temperature. The amount of 5′-DMT-nucleoside loaded on support **9** was evaluated by spectroscopic quantitation of the DMT cation performed as reported above and resulted to be in the range 78−84%. The desired sequence was then completed following standard automated procedures,[25] including final DMT removal, thus obtaining **10**.

Deprotection, Purification, and Characterization of Oligomers a−i. Deprotection and detachment of the synthesized oligomers from resins **7** and **10** were accomplished by an overnight concentrated aqueous ammonia treatment at 50 °C. The crude oligomers were purified by ion exchange HPLC on a Nucleogen DEAE 60-7 Macherey-Nagel column (125 × 4.0 mm, 7 μm); buffer A: 20 mM K_2HPO_4 aqueous solution, pH = 7.0, containing 20% (v/v) CH_3CN; buffer B: 1 M KCl, 20 mM K_2HPO_4 aqueous solution, pH = 7.0, containing 20% (v/v) CH_3CN; a linear gradient from 0% to 100% B in 30 min, flow 1.0 mL/min, was used for oligomers **a**−**h**; a linear gradient from 20% to 100% B in 30 min was employed for the purification of 24-mer **i**. The synthesized oligos showed the following retention times: **a** 25 min 22 s; **b** 22 min 31 s; **c** 22 min 04 s; **d** 21 min 41 s; **e** 21 min 04 s; **f** 22 min 41 s; **g** 24 min 29 s; **h** 22 min 20 s; **i** 24 min 47 s. The isolated oligomers

were desalted by gel filtration on a Biogel P2 column eluted with H_2O/EtOH (4:1).

A purity control on the isolated products was carried out by HPLC analysis on a Vertex LiChrosorb RP18 analytical column (250 × 4.0 mm, 5 μm). Using a linear gradient (from 5 to 50% in 30 min) of CH_3CN in 0.1 M aqueous triethylammonium bicarbonate buffer, pH = 7.0 (flow 0.8 mL/min, detection at λ = 260 nm), 3′-3′ modified oligomers showed the following retention times: **a** 13 min 26 s; **b** 13 min 22 s; **c** 12 min 11 s; **d** 12 min 32 s; **e** 12 min 47 s; **f** 13 min 35 s; **g** 12 min 46 s; **h** 12 min 28 s; **i** 13 min 03 s.

The following compounds were characterized by MALDI-TOF MS: oligonucleotide **b**, mass calculated $[M - H]^- = 4393$; mass observed 4394; oligonucleotide **f**, mass calculated $[M - H]^- = 4681$; mass observed 4684; oligonucleotide **g**, mass calculated $[M - H]^- = 4666$; mass observed 4668; oligonucleotide **h**, mass calculated $[M - H]^- = 4377$; mass observed 4377.

Thermal Denaturation Experiments. The concentration of all the single stranded ODNs was determined spectrophotometrically at 260 nm, using the following molar extinction coefficients for each base:[12] 15400 (A); 11700 (G); 7300 (C); 8800 (T) cm^{-1} M^{-1}.

Quasi-physiological salt conditions (140 mM KCl, 5 mM NaH_2PO_4, 5 mM $MgCl_2$) were used for melting experiments; the resulting aqueous solution was then adjusted to the desired pH values (5.5, 6.0, 6.6, and 7.2) with a diluted NaOH solution. Melting profiles were recorded, realizing a concentration of approximately 1 μM for each strand in 1 mL of the tested solution in Teflon stoppered quartz cuvettes of 1 cm optical pathlength. The resulting mixtures were then allowed to heat at 80 °C for 15 min and then slowly cooled and kept at 5 °C for 20 min. After thermal equilibration at 10 °C, UV absorption at 260 nm was monitored as a function of the temperature, increased at a rate of 0.5 °C/min, typically in the range 10−80 °C. The melting temperatures, reported in Table 1, were determined as the maxima of the first derivative plots of absorbance vs temperature.

Gel Electrophoresis Assays. Nondenaturing polyacrylamide gels (15% polyacrylamide 19:1) were run using an aqueous solution of 89 mM Tris borate, 5 mM $MgCl_2$, 5mM NaH_2PO_4, 0.2 mM EDTA; when required, adjustment to pH = 6.0 was obtained with diluted HCl solutions. The samples were loaded in aqueous 40% sucrose and the gels left at 5 °C and at 45 V for 3 h. Analytical gels were visualized by UV shadowing at 260 nm.

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