A Facile Solid-Phase Synthesis of Oligonucleotides Containing a 3′−3′ Phosphodiester Bond for Alternate Strand Triple-Helix Formation

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An improved protocol for the synthesis of oligonucleotides containing a 3′−3′ phosphodiester linkage, for use in an alternate strand triple-helix formation approach, is reported. Three 16-mers and one 24-mer have been efficiently synthesized and the corresponding triplexes have been analyzed by thermal denaturation experiments and CD spectroscopy.

Introduction

The possibility of using synthetic oligonucleotides as highly specific and efficient regulators of gene expression, targeting DNA sequences by formation of triple-helical structures (antigene approach)[1][2] is extremely appealing. To date, two classes of DNA triplexes have been found, differing in the base composition of the triple-helix forming oligonucleotide (TFO), its orientation relative to the target duplex, and the triplet recognition mode: 1. oligopyrimidines recognize, binding in a parallel orientation, purine tracts of duplex DNA by formation of the isomorphous $C\cdot G\cdot C^+$ and $T\cdot A\cdot T$ Hoogsteen triplets; 2. oligopurines can give stable triplexes by annealing in an antiparallel orientation to the major groove of duplexes if an A (or T) is targeted to an A·T and a G is targeted to a G·C couple by formation of reverse Hoogsteen triplets. Both recognition modes allow targeting only of homopurine stretches in double-stranded nucleic acids; this requirement, associated with the necessity of at least 15−17 bases for a TFO to selectively afford a stable triple-helical complex under quasi-physiological conditions, greatly limits the potential of this technique in pharmaceutical as well as diagnostic applications.

Several research groups[3][4][5][6][7][8][9][10][11][12] have studied the alternate strand triplex approach to broaden the number of possible recognition sites in biologically relevant DNA fragments by sequence-specific triple-helix formation. This implies that adjacent purine domains on alternate strands of W−C duplexes with mixed sequences of the type 5′-$(Pu)_m(Py)_n$-3′ or 5′-$(Py)_m(Pu)_n$-3′ can be simultaneously recognized by oligopyrimidine fragments which, by crossing the major groove, are able to switch strand at the junction between the homopurine and the homopyrimidine domains.

We recently reported[13] on a study of oligopyrimidines containing a 3′−3′ internucleoside phosphodiester linkage as useful triple-helix forming oligonucleotides recognizing 5′-$(Pu)_m(Py)_n$-3′-type duplexes by Hoogsteen triplet formation. Several 15-mers, 16-mers and one 24-mer were synthesized using a convenient solid-phase method, starting from a modified support linking the first nucleotide via the base. Thermal denaturation as well as gel retardation assays were used to check their ability to form intermolecular triplex structures by alternate strand recognition.

In the course of studies aimed at gaining a deeper insight into the sequence effects-stability relationship of such triplexes, particularly focusing on the role played by the inversion site, and at a solution structural characterization of these molecules by ^{1}H-NMR spectroscopy[14], we have explored the possibility of further simplifying the synthetic protocol to obtain oligomers containing a 3′−3′ phosphodiester bond. We describe here an improved solid-phase method that requires only 3′-phosphoramidites as building blocks for the preparation of these molecules, as well as for the formation of the 3′−3′ phosphodiester linkage. Following this strategy, three 16-mers and one 24-mer have been efficiently synthesized and used in triplex formation experiments.

Results and Discussion

In a previous paper[13], we reported on the synthesis of oligodeoxyribonucleotides (ODNs) containing a 3′−3′ internucleoside phosphodiester bond (3′-p-3′) following a synthetic scheme based on the use of functionalized support **5** (Scheme 1), in which the first nucleoside 5′-*O*-DMT-3′-*O*-(2-chlorophenyl)phosphate (**3**), for use in forming the 3′−3′ junction, was attached to the solid-phase matrix via the exocyclic amino function of the cytosine base. This modified support[15][16][17][18] made the 3′-phosphodiester moiety susceptible to nucleophilic attack by the 3′-OH group of a 5′-protected 2′-deoxyribonucleoside, thereby allowing formation of the desired, unnatural 3′−3′ phosphodiester link-

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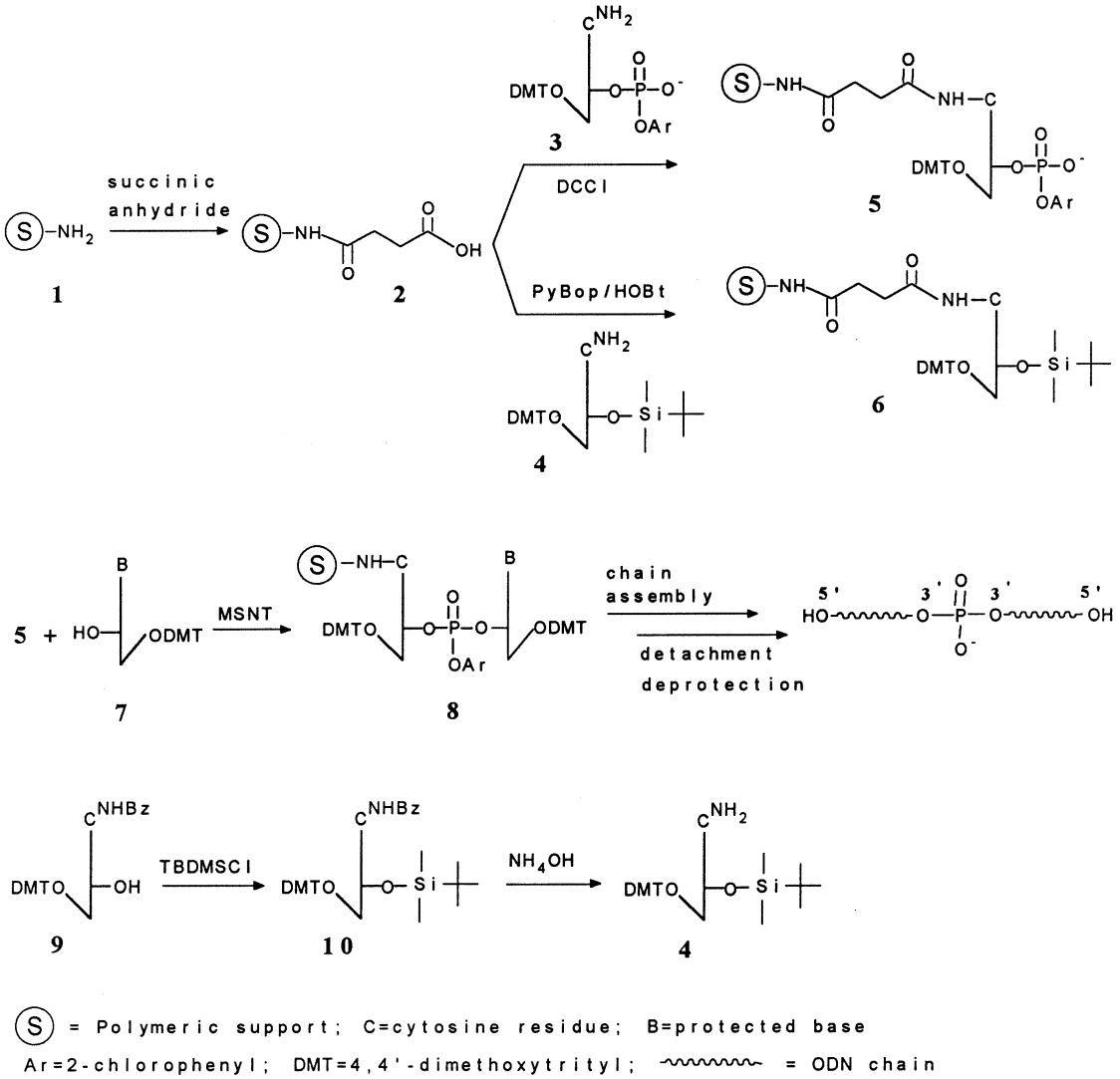
age, and allowing the 5′-OH function, protected as 4,4′-dimethoxytrityl (DMT) ether, to be used for the classical automated chain elongation. The introduction of the 3′-p-3′ inversion of polarity motif could thus be achieved by coupling **5** with a 5′-*O*-DMT-2′-deoxyribonucleoside (**7**) in the presence of MSNT as activator of the phosphodiester moiety.

Scheme 1



This synthetic route for obtaining ODNs containing a 3′−3′ phosphodiester junction necessitated only a 3′-phosphodiester intermediate (**3**) and 3′-phosphoramidites for the chain assembly. This procedure represents, in our opinion, a valuable alternative to the use of both the 3′- and the 5′-phosphoramidites. The latter are in fact currently commercially available, but at a very high cost.

In an effort to produce large quantities of selected sequences under investigation for conformational analysis[14] and calorimetric studies, two drawbacks emerged hampering the extensive applicability of this synthetic route, both connected with the use of functionalized support **5**. Firstly, nucleotide **3** has been reported to be successfully prepared in a single step and in almost quantitative yield by ammonia deprotection of the corresponding *N*-4 benzoyl derivative. Unfortunately, this intermediate, previously employed in the oligonucleotide synthesis based on the phosphotriester approach, is not currently commercially available and therefore needs to be synthesized ad hoc, thus rendering the preparation of **5** quite troublesome. Furthermore, the introduction of the 3′−3′ phosphodiester bond, carried out by treating **5** with **7**, did not proceed with very high yields (typically 78−82%), owing to the intrinsic limited reactivity of phosphodiester functions in heterogeneous reactions with secondary hydroxy groups.

Re-examining the whole aforementioned methodology, we tried to modify our functionalized resin so that the phosphoramidite chemistry could also be exploited in the formation of the 3′−3′ phosphodiester bond. Therefore, as the first unit in the solid support, we considered the possibility of incorporating a nucleoside derivative, the 3′-OH

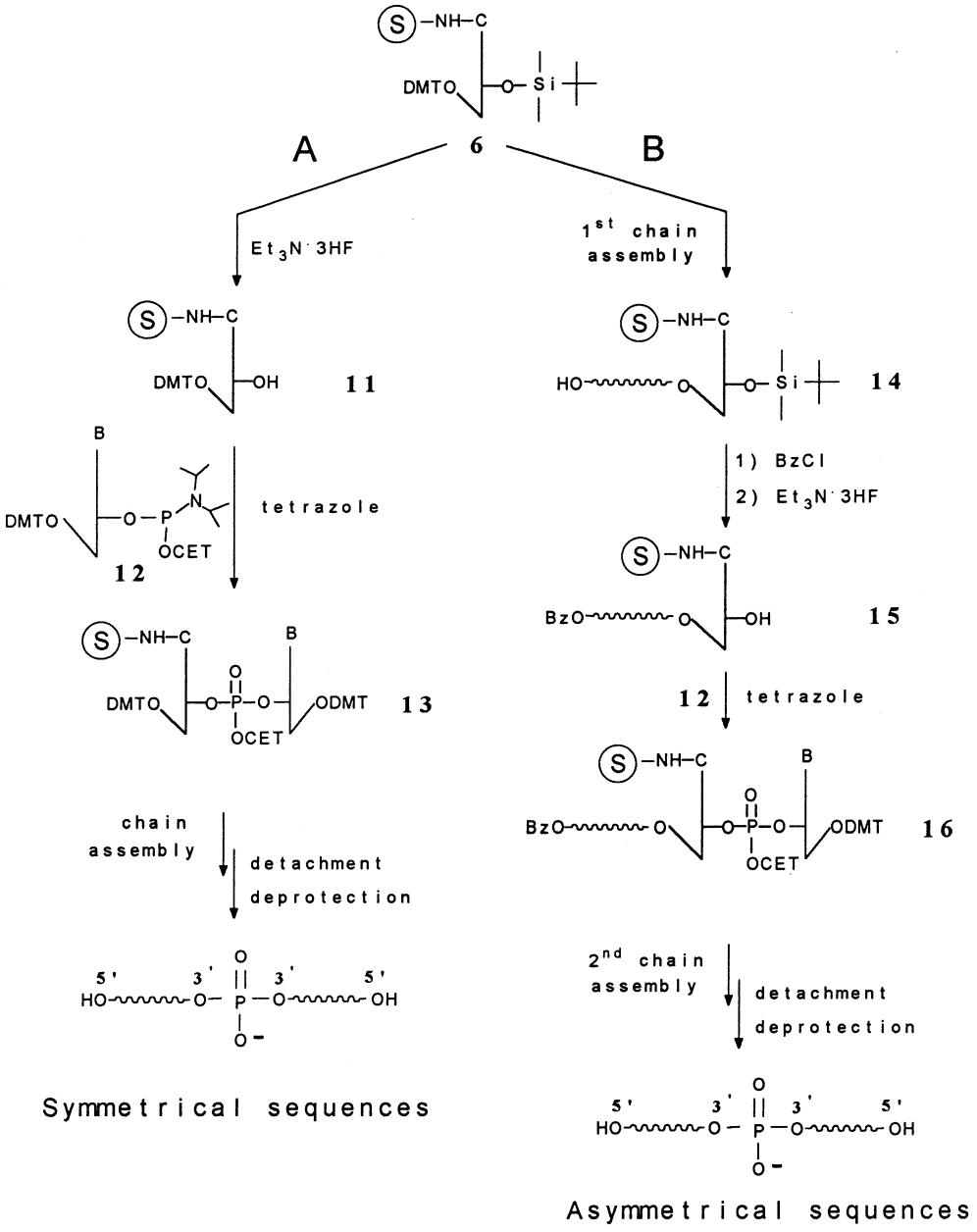
group of which could be reacted, in a standard automated coupling procedure, with a nucleoside 3′-phosphoramidite to give the desired 3′-p-3′ junction.

In this way, a straightforward, high-yield synthetic route was developed, starting from 5′-*O*-DMT-N^4-benzoyl-2′-deoxycytidine (**9**, Scheme 1), which was first converted into the corresponding 3′-protected nucleoside **10**. This was successively deprotected at the cytosine exocyclic amino function by ammonia treatment, giving **4**, which was then treated with succinylated support **2** to afford **6**. Several activators of the carboxy function of support **2** in the reaction with the amino group of nucleoside **4** were tested, such as DCCI, PyBop and TBTU. The best results were obtained using PyBop/HOBt in DMF, which produced functionalized support **6** with a typical loading of 0.12−0.14 meq/g, calculated by quantitative trityl tests. To transiently mask the 3′-hydroxy moiety of nucleoside **9**, the *tert*-butyldimethylsilyl (TBDMS) group was employed in view of its stability under both acidic and mildly basic conditions, as well as during the steps involved in the standard ODN synthesis. In fact, it is a well-documented protecting group of choice for the 2′-OH functions in oligoribonucleotide synthesis[19].

The introduction of the 3′-p-3′ inversion of polarity motif in the ODN chain could thus be achieved:

(i) either as the first step, by removing the TBDMS group and successively reacting **11** with a nucleoside 3′-phosphoramidite (Scheme 2, pathway A); or

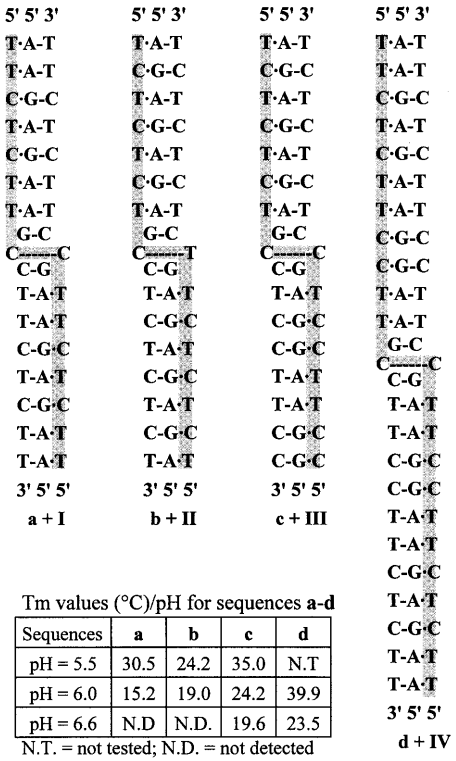
Scheme 2



(ii) after the synthesis of the first 3′−5′-half of the chain, by coupling the 3′-OH end of support **15** with a nucleoside 3′-phosphoramidite (Scheme 2, pathway B).

In the first case, symmetrical sequences with respect to the 3′−3′ junction could conveniently be synthesized, with the chain elongation being performed simultaneously on both the 5′-termini, which allowed the number of coupling steps required for assembly of the whole ODN to be halved. In the other and more general case, where sequences not symmetrical with respect to the 3′−3′ phosphodiester bond were required, the elongation of the 5′-end of support **6** had to be achieved first, followed by final detritylation and a prolonged capping of the terminal 5′-OH group. As outlined in Scheme 2, support **14** was capped at the 5′-OH function and deprotected at the 3′-end, thereby affording **15**. This, coupled with the chosen 5′-*O*-DMT-2′-deoxyribonucleoside-3′-phosphoramidite (**12**), gave support **16**, which was used to synthesize the second 3′-5′-domain of the desired oligomer. Following the aforementioned pathways, symmetrical 16-mers **a** and **b**, asymmetrical 16-mer **c**, and symmetrical 24-mer **d** were synthesized (see Table 1). In both schemes, standard phosphoramidite protocols for the automated ODN synthesis were adopted, with the sole exception that prolonged reaction times were required for the 5′-capping of **14** and for the 3′−3′ coupling. The latter reaction, carried out with the 3′-phosphoramidite derivative of 2′-deoxycytidine, typically proceeded with a 90% yield, while 99% was achieved in the coupling with thymidine 3′-phosphoramidite. A crucial point in the presented synthetic route concerned the removal of the TBDMS protecting group. Several conditions (1 M TBAF solution in THF, TBAF/AcOH, AcOH/H_2O/dioxane), tested on support **6**, led to partial loss of nucleosidic material from the support, presumably due to the high nucleophilicity of fluoride ions, which are able to promote cleavage of the succinate bridge. No such side reaction was observed when using $Et_3N \cdot 3$ HF as reactant (18 h, room temp.), as verified by trityl tests carried out on the resin after this treatment. Conversely, the 5′-OH end of support **14** had to be protected as the benzoic acid ester, rather than by acetylation as in a standard capping procedure[19], since the acetic acid ester proved not to be completely stable to the repeated treatment with fluoride species.

Table 1. Duplex and third strand sequences for the studied alternated strand triplexes and related melting temperatures

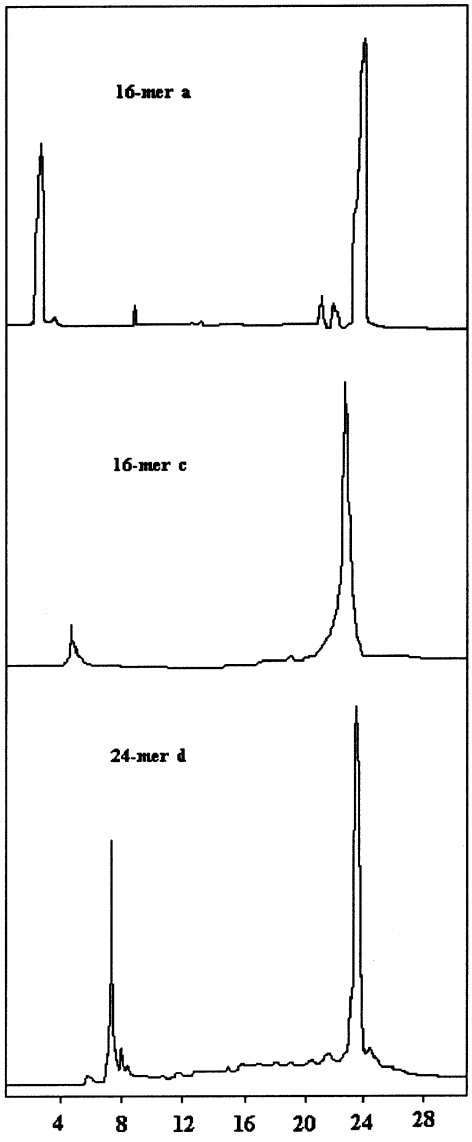


Tm values (°C)/pH for sequences **a-d**

Sequences	a	b	c	d
pH = 5.5	30.5	24.2	35.0	N.T
pH = 6.0	15.2	19.0	24.2	39.9
pH = 6.6	N.D	N.D.	19.6	23.5

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

Figure 1. HPLC profiles of: 16-mer **a**, 16-mer **c**, and 24-mer **d**; column Partisil SAX Whatman, eluent A: 1 mM KH_2PO_4, 20% CH_3CN, pH = 7.0; eluent B: 350 mM KH_2PO_4, 20% CH_3CN, pH = 7.0; gradient from 0−100% B in 30 min for 16-mer **a** and 16-mer **c**; gradient from 20−100% B in 30 min for 24-mer **d**; flow rate 0.8 ml/min



Standard ammonia treatment (12 h, 50°C) allowed detachment from the support and the total deprotection of oligomers **a**−**d**, which were analyzed by HPLC on an anion-exchange Partisil 10 SAX column (see Figure 1) and purified on a DEAE ion-exchange column. The isolated oligomers were successively desalted on a Biogel P2 column and their purity was checked by reversed-phase HPLC analysis. For 16-mers **a**, **b** and **c**, the yields of isolated products were always comparable, thus indicating that pathways A and B guarantee very similar synthetic efficiencies.

Thermal denaturation experiments were carried out by mixing 3′−3′ ODNs **a**, **b**, **c** and **d** with the corresponding target duplexes (**I**, **II**, **III** and **IV**, respectively) in a 1:1 ratio at a concentration of approximately 1 μM for each strand in a 140 mM KCl, 5 mM NaH_2PO_4, 5mM $MgCl_2$ solution adjusted to various pH values (5.5, 6.0, 6.6 and 7.2). Melting curves performed at acidic pH showed a typical biphasic behavior, with the first sigmoid attributable to the triplex dissociations, while at higher temperatures the expected pH-insensitive duplex-to-coil transitions could be observed. As an example, the thermal denaturation profile of the complex formed by oligomer **d** and duplex **IV** is shown in Figure 2. As found previously[13], comparing the melting temperatures relative to the triple-helical complexes formed by 16-mers **a**, **b** and **c**, a strong sequence-dependent stability is apparent (see Table 1).

Triplex formation ability of 24-mer **d** was also investigated by CD spectroscopy, which is a highly suitable technique for distinguishing between DNA structures. An equimolar mixture of **d** and duplex **IV**, dissolved in the same buffer as used for the UV melting studies, at pH = 7.5 and 10°C, showed only the profile due to the duplex form. On the other hand, at pH = 6.0 and 10°C, a CD spectrum consistent with a typical triple-stranded DNA[4][7][20], with a maximum at 280 nm, a crossover at 262 nm, and two minima (a strong negative band at 212 nm and another minimum at 250 nm, see Figure 3), was obtained.

Figure 3. CD spectra of duplex **IV** (-----) and of the triplex formed by **d** + **IV** (− − −) in 140mM KCl, 5 mM NaH_2PO_4, 5 mM $MgCl_2$, pH = 6.0 at 10°C

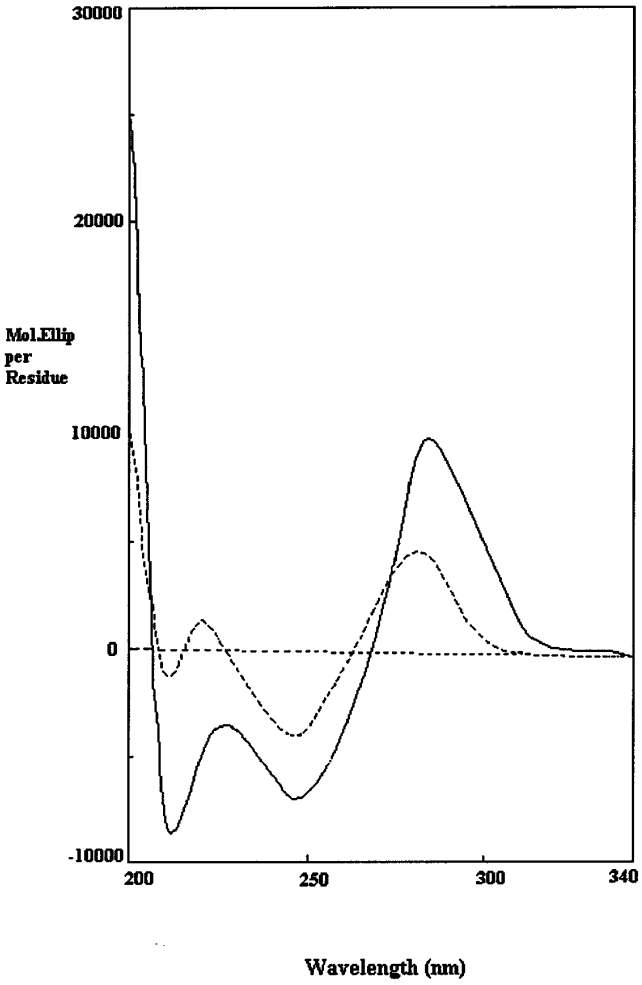
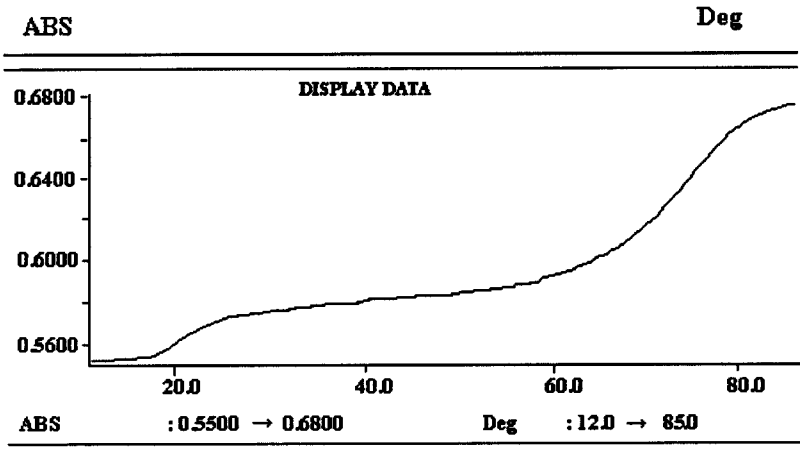


Figure 2. Melting profile of oligomer **d** with target duplex **IV** at pH = 6.6; buffer: 5 mM $MgCl_2$, 140 mM KCl, 5 mM NaH_2PO_4; $c \approx 1$ μM each strand; 0.5°C/min



Conclusions

A new derivatized support, **6**, functionalized with a base-linked nucleoside having the 5′-OH and the 3′-OH functions orthogonally protected by DMT and TBDMS groups, respectively, has been efficiently prepared. Conditions were found for the total, selective removal of the TBDMS group from the solid phase in the presence of a DMT-protected 5′-end and a succinic bridge linking the first nucleosidic base to the support. This resin allowed us to develop an improved synthetic procedure for obtaining oligodeoxyribonucleotides containing a 3′−3′ phosphodiester bond as TFO-targeting duplexes of the type $5'(Pu)_m(Py)_n3'$, by using only nucleoside 3′-phosphoramidites, both in the chain assembly and in the formation of the 3′−3′ phosphodiester junction. UV thermal analysis and CD experiments showed that these molecules are able to give intermolecular triplex structures by cooperative, alternate strand recognition of adjacent stretches of homopurines in the target duplex.

In view of the fact that several conjugating molecules are commercially available as 3′-phosphoramidite derivatives, the possibility of using support **6** as a versatile and convenient precursor for a number of 3′-conjugated ODNs (or 3′,5′-diconjugated) is currently under investigation in our laboratories.

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Experimental Section

Materials and Methods: Tentagel resin was purchased from Rapp Polymere, Tübingen, Germany. The solid support functionalizations were carried out in short glass columns (5 cm length, 1 cm i.d.) equipped with a sintered glass filter, a stopcock, and a cap. − The oligonucleotides were assembled with a Millipore Cyclone Plus DNA synthesizer, using commercially available 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite nucleosides as building blocks. − HPLC analyses were carried out with a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. − UV measurements were performed with a Perkin-Elmer Lambda 7 spectrophotometer. − Thermal denaturation experiments were run with a Cary 1E Varian spectrophotometer equipped with a Haake PG20 thermoprogrammer at λ = 260 nm. − NMR spectra were recorded with Bruker WM-400 and WM-270 spectrometers. All chemical shifts are expressed as δ values with respect to the residual solvent signal. − CD spectra were recorded with a Jasco J-715 spectropolarimeter. − Abbreviations: DCCI = *N*,*N*′-dicyclohexylcarbodiimide; PyBop = benzotriazol-1-yl-oxytripyrrolidinephosphonium hexafluorophosphate; TBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; HOBt = 1-hydroxybenzotriazole; TBAF = tetrabutylammonium fluoride; DIEA = *N*,*N*-diisopropylethylamine.

Synthesis of Nucleoside **4**: 800 mg (1.26 mmol) of commercially available N^4-benzoyl-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-deoxycytidine (**9**) was treated with 265 mg (1.76 mmol) of *tert*-butyldimethylsilyl chloride and 160 mg (2.35 mmol) of imidazole in 4 ml of anhydrous DMF. After 12 h, TLC analysis (eluent $CHCl_3$/CH_3OH, 95:5, v/v) showed the complete conversion of the starting material into the 3′-protected derivative **10** (R_f = 0.75). The reaction mixture was concentrated under reduced pressure, the residue was redissolved in $CHCl_3$, and the resulting solution was washed twice with satd. $NaHCO_3$ solution. The organic phase was concentrated to dryness and then treated with conc. aq. ammonia/CH_3OH (1:1, v/v) for 24 h at 50°C under stirring. The solution was concentrated under reduced pressure and the crude product was chromatographed on a silica-gel column, eluting with increasing amounts of CH_3OH in $CHCl_3$ containing 0.5% pyridine. Fractions eluted with $CHCl_3$/CH_3OH, 9:1 (v/v), collected and concentrated to dryness, afforded 745 mg (1.16 mmol, 92% yield starting from **9**) of desired *N*-deprotected nucleoside **4**.

10: ^{1}H NMR ($CDCl_3$): δ = 8.38 (d, 1 H, 6-H), 7.88−6.83 (complex signals, 19 H, aromatic protons and 5-H), 6.25 (dd, 1 H, 1′-H), 4.45 (m, 1 H, 3′-H), 4.02 (m, 1 H, 4′-H), 3.80 (s, 6 H, OCH_3 of the DMT residue), 3.43 (AB part of an ABX system, 2 H, 5′-H_2), 2.39 (m, 2 H, 2′-H_2), 0.81 [s, 9 H, $(CH_3)_3C$], 0.01 and −0.05 (2 s, 3 H each, 2 CH_3Si). − ^{13}C NMR ($CDCl_3$): δ = 169.82 (exocyclic amide carbonyl), 163.37 (C-4), 162.06 (C-2), 158.58, 144.33, 143.90, 135.16, 129.94, 129.50, 128.10, 127.83, 127.01 and 113.15 (aromatic carbons), 114.09 (C-6), 96.27 (C-5), 86.83, 86.76 and 86.56 (C-1′, C-4′ and quaternary carbon atom of the DMT residue), 70.38 (C-3′), 61.79 (C-5′), 55.09 (2 OCH_3 of the DMT residue), 42.07 (C-2′), 25.53 [$(CH)_3C$], 18.35 [$(CH)_3C$], −4.76 and −5.14 [$(CH_3)_2Si$].

4: ^{1}H NMR (CD_3OD): δ = 8.00 (d, 1 H, 6-H), 7.86−6.81 (complex signals, 13 H, aromatic protons), 6.22 (dd, 1 H, 1′-H), 5.66 (d, 1 H, 5-H), 4.48 (m, 1 H, 3′-H), 3.92 (m, 1 H, 4′-H), 3.73 (s, 6 H, OCH_3 of 4,4′-dimethoxytrityl residue), 3.41 (AB part of an ABX system, 2 H, 5′-H_2), 2.23 (m, 2 H, 2′-H_2), 0.81 [s, 9 H, $(CH_3)_3C$], 0.01 and −0.05 (2 s, 3 H each, 2 CH_3Si). − ^{13}C NMR (CD_3OD): δ = 167.69 (C-4), 158.19 (C-2), 160.42, 146.11, 142.47, 136.89, 131.59, 129.61, 129.14 and 114.45 (aromatic carbons), 114.89 (C-6), 96.14 (C-5), 88.26, 87.87 and 87.54 (C-1′, C-4′ and quaternary carbon atom of the DMT residue), 72.63 (C-3′), 63.67 (C-5′), 56.00 (2 OCH_3 of the DMT residue), 43.06 (C-2′), 26.52 [$(CH)_3C$], 19.00 [$(CH)_3C$], −4.14 and −4.38 [$(CH_3)_2Si$]. − FAB MS: *m*/*z* = 644 [M + H]$^+$.

Functionalization of Tentagel Resin − Preparation of Support **6**: A mixture of 1.0 g of support **1** (0.24 mmol of amino groups per g) and 1.0 g (10 mmol) of succinic anhydride in 8 ml of anhydrous pyridine was shaken for 16 h at room temp. The resulting support **2** was filtered off, exhaustively washed with pyridine, $CHCl_3$ and Et_2O, and dried under reduced pressure. It was then reacted with 655 mg (0.96 mmol) of **4**, dissolved in 4 ml of anhydrous DMF, in the presence of 270 mg (0.5 mmol) of PyBop, 70 mg (0.5 mmol) of HOBt and 170 μl of DIEA for 16 h at room temp. under shaking. The final support **6** was washed with $CHCl_3$, CH_3OH, and Et_2O, and then dried under reduced pressure. Loadings of 0.12−0.14 meq/g of the 5′-DMT-2′-deoxynucleoside 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 upon acidic treatment (70% $HClO_4$/EtOH, 3:2, v/v) of a weighed amount of the dried support.

Chain Assembly of Symmetrical Oligomers **a**, **b** *and* **d** (*Pathway* **A**): For the preparation of ODNs **a**, **b** and **d**, the following procedure was used: 50 mg (0.0065 meq) of functionalized support **6** were left in contact with 80 μl (0.49 meq) of $Et_3N \cdot 3$ HF in 450

μl of anhydrous THF for 18 h at room temp. After washing, the resulting support **11** was treated, according to an automated coupling procedure, with a 1:1 (v/v) solution of 5′-DMT-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite-N^4-benzoyl-2′-deoxycytidine − or -thymidine − (**12**, 0.1 M) and tetrazole (0.1 M) in anhydrous acetonitrile for 2 h at room temp. Incorporation yields of the second nucleotide residue forming the 3′−3′ junction in support **13**, evaluated by DMT tests, were found to be typically 90% for the coupling with the 2′-deoxycytidine monomer, and 99% in the case of the thymidine derivative. Chain elongation on both the 5′-ends of polymer **13** was then carried out using longer coupling cycles (8 min) and higher phosphoramidite concentrations (45 mg/ml) than in standard automated procedures[19], leading to coupling yields consistently in excess of 98% per cycle.

Chain Assembly of Asymmetrical Oligomer **c** (*Pathway* **B**): The synthesis of sequence **c**, asymmetrical with respect to the 3′-p-3′ phosphodiester bond, was typically carried out starting from 50 mg (0.0065 meq) of derivatized support **6**. After assembly of the first domain of the ODN chain according to standard automated methods[19], the final DMT group was removed and a prolonged (30 min) treatment with 0.5 ml of benzoyl chloride in pyridine (0.5 ml) was performed to block the 5′-end. Removal of the TBDMS protecting group was achieved by $Et_3N \cdot 3$ HF treatment, as described above. The resulting support **15** was successively coupled, by a standard automated procedure[19], with the thymidine 3′-phosphoramidite derivative. The desired sequence was then completed following standard automated procedures[19], including final DMT removal. Coupling yields in the range 98−99% were invariably obtained for the chain assembly, as verified by DMT tests.

Deprotection, Purification and Characterization of Oligomers **a−d**: The synthesized oligomers were deprotected and detached from the solid supports by an overnight treatment with conc. aq. ammonia at 50°C. The supernatant was filtered and the support was washed with water. The combined filtrate and washings were concentrated to dryness, the residue was redissolved in water, and analyzed by HPLC on a Partisil 10 SAX column (Whatman, 4.6 × 250 mm, 7 μm) eluting with linear gradients of KH_2PO_4 (20% CH_3CN, pH = 7.0) from 1 to 350 mM in 30 min, flow rate 0.8 ml/min. Purification of the crude materials was performed 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 aq. solution, pH = 7.0, containing 20% (v/v) CH_3CN; buffer B: 1 M KCl, 20 mM K_2HPO_4 aq. solution, pH = 7.0, containing 20% (v/v) CH_3CN; a linear gradient from 0−100% B in 30 min, flow rate 0.8 ml/min, was used for oligomers **a−c**; a linear gradient from 20−100% B in 30 min was employed for the purification of 24-mer **d**. The isolated oligomers were desalted by gel filtration on a Biogel P2 column, eluting with H_2O.

A purity control on the isolated products was carried out by HPLC analysis on a Partisphere Whatman RP18 analytical column (125 × 4.0 mm, 5 μm). Using a linear gradient (from 5−40% in 30 min) of CH_3CN in 0.1 M aq. triethylammonium bicarbonate buffer, pH = 7.0 (flow rate 0.8 ml/min, detection at λ = 260 nm), the 3′−3′ modified oligomers proved to be more than 98% pure, showing the following retention times: **a** 11 min 42 s, **b** 12 min 19 s, **c** 11 min 35 s, **d** 12 min 52 s.

The following compounds were characterized by MALDI-TOF MS: oligonucleotide **c**, calculated mass for $[M - H]^-$ 4666; observed mass 4668; oligonucleotide **d**, calculated mass for $[M - H]^-$ 7084; observed mass 7085.

Thermal Denaturation Experiments: The concentrations of the synthesized ODNs were determined spectrophotometrically at λ = 260 nm and at 85°C, using the following molar extinction coefficients for each base[8]: 15400 (A), 11700 (G), 7300 (C), 8800 (T) $cm^{-1}M^{-1}$.

A 140 mM KCl, 5 mM NaH_2PO_4, 5 mM $MgCl_2$ solution was used for melting experiments, which were carried out at different pH values (5.5, 6.0, 6.6 and 7.2). Melting curves 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 path length. The solutions were heated at 80°C for 15 min, and then were slowly allowed to cool and kept at 5°C for 20 min. After thermal equilibration at 10°C, UV absorption at λ = 260 nm was monitored as a function of the temp., which was increased at a rate of 0.5°C/min, typically over the range 10−80°C. The melting temperatures, reported in Table 1, were determined as the maxima of the first derivative of absorbance-vs.-temperature plots.

Circular Dichroism Spectroscopy: Buffer and sample conditions were the same as those used for the UV thermal analysis studies. Optical cells with path lengths of 0.1 cm were used. CD spectra were recorded at 10°C, with the temp. kept constant by means of a circulating water bath. Throughout all the experiments, nitrogen was continuously circulated through the cuvette compartment.

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