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Synthesis and Characterization of new 3'-3' linked Oligodeoxyribonucleotides for Alternate Strand Triple Helix Formation

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Abstract. Protected forms of 1,2,3-propanetriol and *cis*, *cis*-1,3,5-cyclohexanetriol were incorporated onto solid supports which were exploited in the solid phase synthesis of 3'-3' linked oligodeoxyribonucleotides (ODNs), involving only nucleoside 3'-phosphoramidites as building blocks. UV thermal denaturation analysis showed the ability of ODNs with this inversion of polarity motif to cooperatively hybridize with duplexes of the type 5'-$(Pu)_m(Py)_n$-3' in an alternate strand recognition approach.

Introduction.

Shortly after the discovery of the structure of double helical DNA, Felsenfeld and coworkers found that RNA and DNA homopolymers can, under certain salt and pH conditions, also give rise to triple stranded complexes.[1,2] However, only recently have triplexes been thoroughly investigated in view of their applications in molecular biology and diagnostics and, most noticeably, in the *antigene strategy* as new and universal therapeutic agents.[3,4,5] Synthetic oligodeoxyribonucleotides of defined sequences can bind to the major groove of double stranded DNA having oligopurine-oligopyrimidine tracts, forming local triple helices by sequence-specific hydrogen bonding: pyrimidine-rich triplex forming oligonucleotides (TFOs) target in a parallel orientation homopurine fragments in double stranded DNA by Hoogsteen T•AxT and C•GxC$^+$ triplets formation, while homopurines can recognize in an antiparallel orientation the purine strand of the Watson-Crick duplex upon generation of T•AxA and C•GxG triads.[#] Therefore considerable activity has been directed to the exploitation, either *in vitro* or *in vivo*, of TFOs as modulators of gene expression since they can compete with the binding of proteins and inhibit the transcription of a specific gene.

The efficiency of the TFO binding is affected by several factors, such as pH, cations concentration,

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composition and length of the third strand; the major requirement for a stable triplex is a fairly long (at least 16-17 bases) homopurine tract in the duplex DNA, which hardly reduces the number of potentially recognizable sequences of biological interest. In an effort to extend the range of triplex forming DNA targets, Horne and Dervan proposed the alternate strand approach,[6] whereby ODNs hybridize simultaneously with the adjacent purine tracts on alternate strands of (purine)$_m$(pyrimidine)$_n$ sequences by switching strands at the oligopurine-oligopyrimidine junction of the duplex DNA. If the third strand is able to efficiently cross the major groove at this junction without destabilizing the resulting triplex, the TFO can tightly and cooperatively bind to adjacent purine tracts on alternate strands of the duplex. For the canonical pyrimidine triple helix recognition mode, based on Hoogsteen triplet formation, to be effective, the selected TFO must have an appropriate inversion of polarity at the junction between the homopurine and the homopyrimidine sequences of the duplex to ensure the required orientation of the third strand parallel to each homopurine domain. Topologically, this necessitates a 3'-3' (or a 5'-5') internucleoside linkage connecting the two 3'- (or 5'-) ends of the TFO.

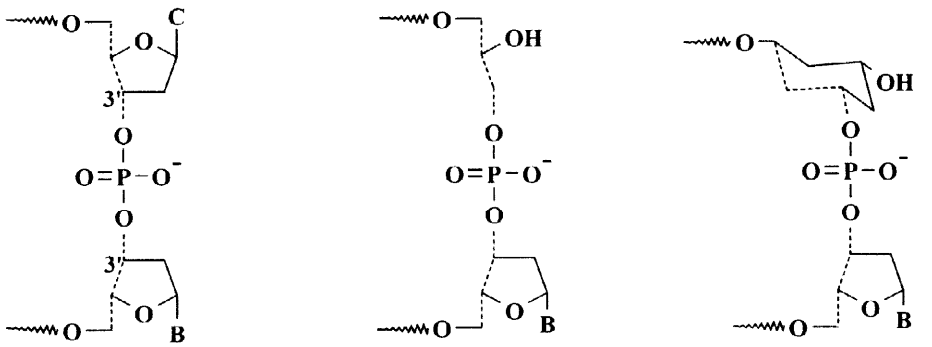
Several internucleoside junctions have been proposed as linkers for alternate strand TFOs; but while 5'-5' tethers[7] do not impart a strong cooperativity in the binding of the two 3'-5' fragments constituting the third strand, many examples of 3'-3' linked ODNs have been shown to form stable alternate strand (or "switched") triplexes. Horne and Dervan[6] successfully tested a 1,2-dideoxy-D-ribose unit as linker connecting the 3'-phosphate ends of two homopyrimidine 9-mers, thus obtaining a modified oligonucleotide containing a 3'-3' junction which recognized a 5'-(purine)$_9$NN(pyrimidine)$_9$-3' sequence of duplex DNA, where NN was the dinucleotide GC facing the inversion site of the third strand. In a subsequent study, Ono and coworkers[7] synthesized TFOs containing two 1,3-propanediol units inserted, in the form of a phosphate diester, as 3'-3' linker, which resulted in a highly stabilized triplex. Molecular modeling studies suggested the design of an *o*-xylenyl unit to join the 3'-OH ends of two 9-mers, demonstrated by Froehler and his group[8,9] to provide an appropriate length and conformational constraint, with a minimum of steric crowding at the inversion site. A slightly different approach has been pursued by Asseline and Thuong,[10,11,12] who described $C^{3'}$-$^{3'}C$ and $C^{3'}$-$^{3'}U$ dinucleosides linked through their bases *via* a polymethylene bridge.

Keeping in mind the unanswered question whether and to what extent the bases present in the 3'-3' linked dinucleotide would effectively contribute to the stability and specificity of the TFO binding, we recently[13,14] focused our attention on oligonucleotides containing a 3'-3' internucleoside phosphodiester bond and their ability to form switched triplexes. By comparison of the melting temperatures of a number of triple helical complexes formed by third strands differing only by point modifications, a small, but detectable, influence in the recognition process of the bases at the inversion site and, more evidently, of those flanking the 3'-3' linked dinucleotide box, was found.

We here describe the synthesis and characterization of ODNs containing a 1,2,3-propanetriol or a *cis*, *cis*-1,3,5-cyclohexanetriol residue replacing one nucleoside unit at the inversion site in oligomers containing 3'-3' phosphodiester bonds (Figure 1). To better interpret their affinity towards duplexes of the type 5'-(purine)$_m$(pyrimidine)$_n$-3', measured by UV thermal analysis, in terms of the structural characteristics of the

inversion of polarity motif, the same nucleotide sequences were synthesized and compared to all-nucleotide 15-mers and 16-mers having a $C^{3'}$-p-$^{3'}C$ or a $C^{3'}$-p-$^{3'}T$ inversion bridge (see Table 1).

FIGURE 1



Internucleosidic 3'-3' phosphodiester junction

3'-3' glycerine - containing junction

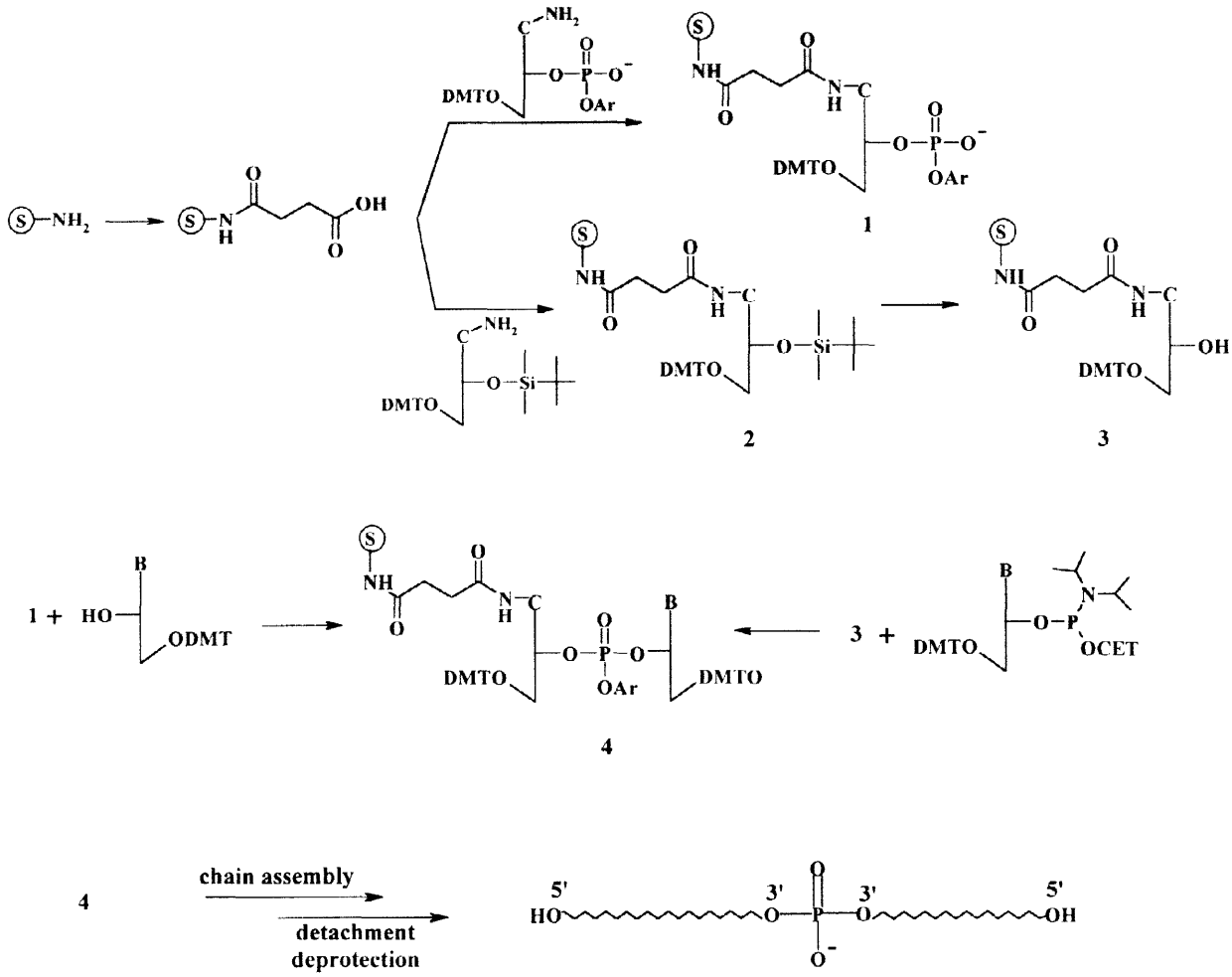
3'-3' cyclohexanetriol - containing junction

Results and Discussion.

The synthesis of oligonucleotides with a 3'-3' phosphodiester linkage had been previously reported by H-phosphonate chemistry and required the use of 3'-DMT protected nucleoside 5'-H-phosphonates as well as the commercially available 5'-DMT-nucleoside 3'-H-phosphonates.[15] Our method is based on the usage of a solid support linking the first nucleoside through the base (**1**[13] or **3**,[14] Scheme 1), so that its 3'-O-(2-chlorophenyl)phosphate (or 3'-OH) end could be directly exploited to form the 3'-3' phosphodiester linkage, while the 5'-OH was used for the automated oligonucleotide assembly, following a classical phosphoramidite protocol.[16] Instead of a monodirectional elongation of the chain, a bidirectional approach could be thus introduced, through two possible synthetic routes which required only nucleoside 3'-phosphoramidites as building blocks. For the synthesis of sequences symmetric with respect to the inversion site, the coupling of **1** or **3** with the chosen 5'-DMT-nucleoside or 5'-DMT-nucleoside 3'-phosphoramidite, respectively, creating the 3'-3' linked dinucleotide unit **4**, was carried out as the first step, followed by the oligonucleotide assembly, with the chain growing at both 5'-ends simultaneously. This method was particularly convenient as it reduced by half the number of couplings necessary to complete the desired sequence. In the more general case where sequences not symmetric with respect to the inversion site were required, the assembly of the first 3'-5' half of the oligonucleotide was performed on **1**[13] or **2**[14] by standard procedures,[16] followed by capping of the 5'-OH end, coupling of the deprotected 3'-end with the 5'-DMT-nucleoside (or 5'-DMT-nucleoside-3'-phosphoramidite) to produce the 3'-3' phosphodiester bond and, finally, synthesis of the second 3'-5' half of the chain.

Considering that in the presented synthetic scheme the first nucleotide (or nucleoside) base anchors the

SCHEME 1



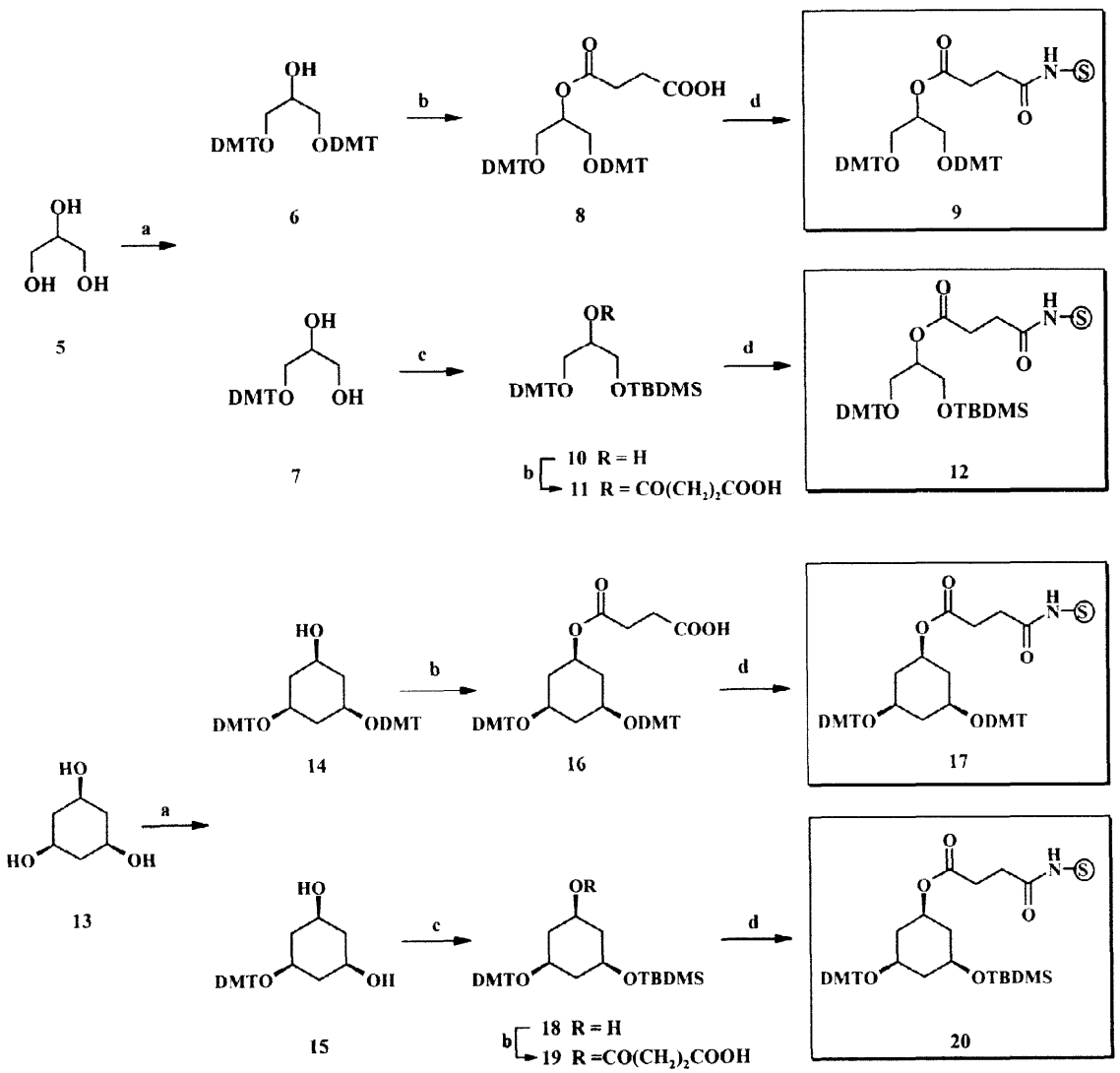
Ⓢ = Polymeric support; C^{NH_2} = cytosine residue; DMT = 4,4' - dimethoxytrityl; Ar = 2 - chlorophenyl; B = protected base; CET = 2 - cyanoethyl; ∿∿∿ = ODN chain.

ODN to the solid support while the sugar acts as a three-carbon atoms spacer between the two 3'-phosphodiester ends of two oligomers, we thought of substituting this first residue with trifunctional molecules which, once incorporated in the solid phase, could be used as inversion of polarity motif for 3'-3' linked oligonucleotides. As depicted in Figure 1, 1,2,3-propanetriol and *cis*, *cis*-1,3,5-cyclohexanetriol can conveniently mimic the ribose unit of a nucleoside, having a three-carbon atom skeleton separating the hydroxyls in 1,3 position and a residual hydroxyl group available to attach the molecule to the solid support. These two compounds, commercially available at low cost, have been chosen as representative examples of spacers to be used as 3'-3' linkers, exhibiting a high degree of flexibility, if compared to a nucleoside residue.

For the incorporation in the solid matrix, 1,2,3-propanetriol and *cis*, *cis*-1,3,5-cyclohexanetriol had to be conveniently protected and functionalized as reported in Scheme 2. The general synthetic strategy for the

synthesis of sequences symmetric with respect to the 3'-3' linkage involved the use of the triols identically protected at two hydroxyl functions as DMT-ethers and successively derivatized with succinic anhydride at the secondary hydroxyl. Alternatively, an orthogonal protection (DMT associated with the TBDMS group) of two hydroxyl moieties in the starting substrates, followed by succinylation of the remaining hydroxyl group, was required for the preparation of asymmetric sequences. As a result of the introduction of different substituents on prochiral molecules, in the latter case, chiral racemates of the protected triols are produced, which is reflected in the final isolation of the desired ODNs as a mixture of two diastereomers.

SCHEME 2



a) DMTCl / pyridine, b) succinic anhydride / pyridine
c) TBDMSCl / imidazole / DMF d) Tentagel resin / DCCI / CH_2Cl_2

Glycerine (**5**), by reaction with 2.0 eq of DMTCl, was converted into compounds **6** and **7**, in 65 % and 25

% yields, respectively. These stoichiometric ratios, even if not assuring the complete disappearance of the starting material, allowed the best yields of the desired compounds; in fact, when using higher amounts of the tritylating agent, the tritritylated derivative was recovered as the main product. The secondary hydroxy function of compound **6** was then succinylated by addition of succinic anhydride furnishing **8** (90 % yield), which was subsequently reacted with an amino-functionalized polymeric matrix (Tentagel, 0.22 meq/g) in the presence of DCCI as carboxyl group activator, giving derivatized support **9**. The incorporation in the solid phase of the bis-dimethoxytritylated glycerine residue was 0.18 meq/g (82 %), as evaluated by spectroscopic measurements of the 4,4'-dimethoxytrityl cation released by acidic treatment of a weighed amount of resin **9**. Reaction of **7** with TBDMSCl/imidazole in DMF furnished in almost quantitative yields **10**, then succinylated affording **11** in 82 % yield. As described for **8**, **11** was treated with the Tentagel resin giving support **12** (0.18 meq/g, 82 % incorporation when using DCCI as condensing reagent). The same synthetic pathways starting from cyclohexanetriol **13** were followed to give derivatized supports **17** and **20**, with functionalizations of 0.16 meq/g (73 % incorporation) and 0.15 meq/g (68 % incorporation), respectively. Several activators (TBTU, DCCI, PyBop, 2-chloro-1-methylpyridinium iodide) were tested in the coupling of the succinylated compounds with the primary amino function of the polymeric support, the best yields being always obtained with DCCI. The identity and purity of all the synthesized compounds were ascertained by ^{1}H-, ^{13}C-NMR and FAB MS data.

TABLE 1 Synthesized 3'– 3' oligonucleotides

a	**5'TCTCTCT3'-p-(glycerine)-p–3'TCTCTCT5'**
b	**5'TCTCTCT3'-p-(cyclohexanetriol)-p-3'TCTCTCT5'**
c	**5'TCTCTCT3'-p-(glycerine)-p–3'CTCTCTCT5'**
d	**5'TCTCTCT3'-p-(cyclohexanetriol)-p-3'CTCTCTCT5'**
e	**5'TCTCTCTC3'-p-3'TCTCTCT5'**
f	**5'TCTCTCTC3'-p-3'CTCTCTCT5'**
g	**5'A3'–p-(glycerine)-p-3'A5'**
h	**5'A3'–p-(cyclohexanetriol)-p-3'A5'**

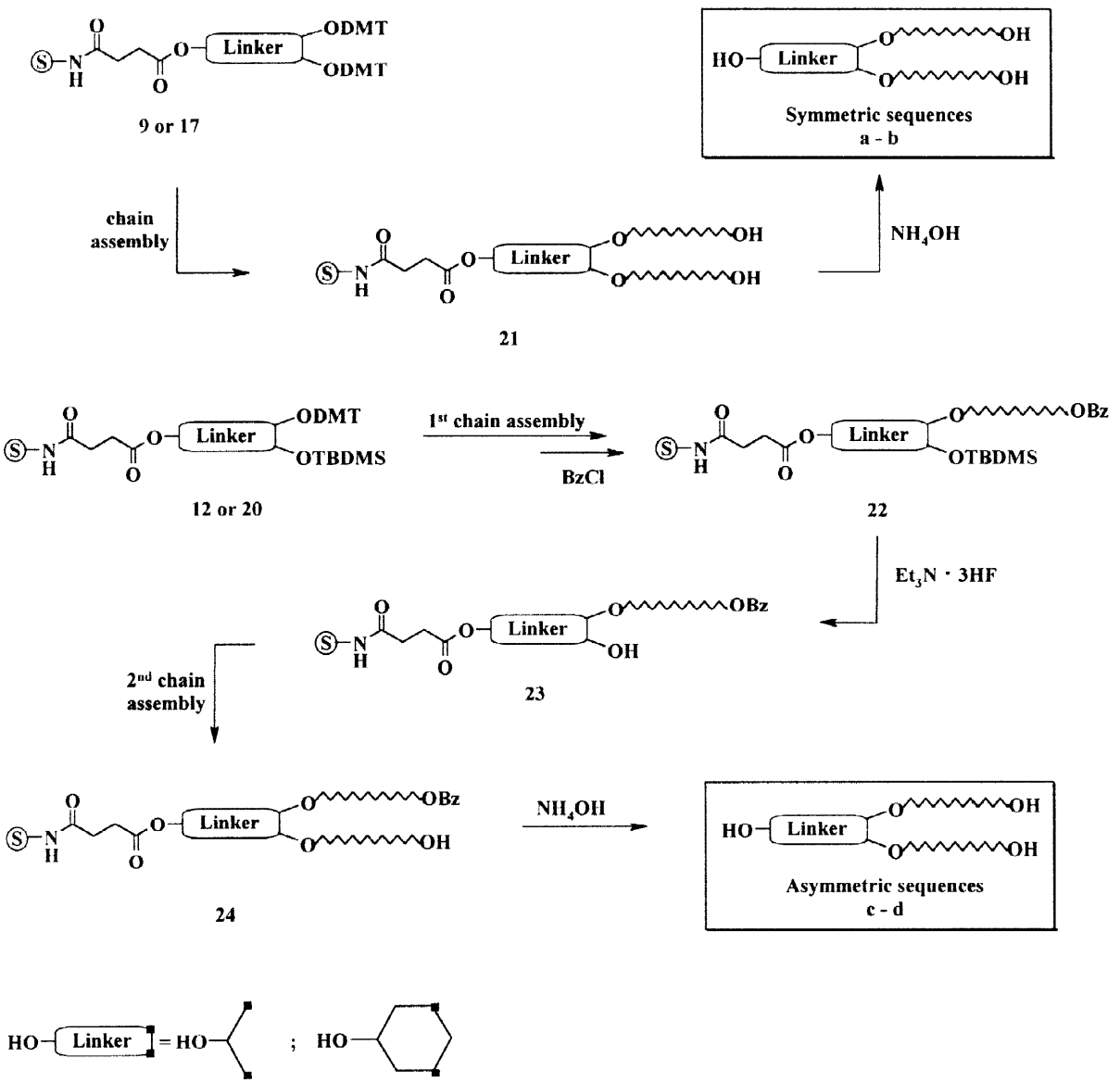
p = phosphodiester junction

To check the correct growth of the ODN chain on supports **9**, **12**, **17** and **20**, adenosine dinucleoside 3'-phosphate derivatives **g** and **h** (Table 1), containing a glycerine or a cyclohexanetriol residue, respectively, as 3'-3' linker, were synthesized and characterized by ^{1}H-, ^{31}P-NMR and FAB mass spectrometry. Comparable overall yields of final dinucleotide products **g** and **h**, independently from the route followed, were found, as evaluated by HPLC analysis of the crude detached materials; a symmetric synthesis route (Scheme 3) was used starting from supports **9** or **17**, while, following the asymmetric approach, also described in Scheme 3, supports **12** or **20** were exploited. To test the feasibility of the proposed synthetic pathways, an overnight ammonia treatment was carried out on supports **9**, **12**, **17** and **20**, which in all cases gave derivatives **6**, **10**, **14** and **18**,

respectively, as the sole and totally detached products, as judged by DMT tests performed on the dried supports, and by TLC and ^{1}H-NMR analysis of the released materials.

Chain assembly of symmetric sequences **a** and **b** ($^{5'}$TCTCTCT$^{3'}$-linker-$^{3'}$TCTCTCT$^{5'}$) was carried out on supports **9** or **17** on a 5 μmol scale using a classical phosphoramidite protocol.[16] Seven coupling steps in both cases were performed using commercially available 2'-deoxyribonucleoside 3'-phosphoramidites as building blocks in 45 mg/mL concentration; coupling efficiencies were always superior to 98 %, as checked by DMT tests in the DCA treatments.

SCHEME 3



On the other hand, asymmetric sequences **c** and **d** ($^{5'}$TCTCTCT$^{3'}$-linker-$^{3'}$CTCTCTCT$^{5'}$) were prepared starting from supports **12** or **20**, respectively. The first 3'-5' fragment was assembled in a standard manner, with

final DMT removal; the 5'-OH end of the chain was then capped by addition of benzoylchloride in pyridine (1:1, v/v, r.t., 30 min), affording **22**, followed by TBDMS removal by treatment with $Et_3N{\cdot}3HF$ (18 h, r.t.). This reagent was preferred to other desilylating systems, since it allowed complete deprotection of the hydroxyl function linked to the solid phase avoiding side reactions (both the benzoic ester at the 5'-end and the succinic bridge were completely stable even after a prolonged treatment). Conversely, when using the most commonly used TBAF reagent[17] (1.1 M solution in THF or mixtures TBAF/AcOH) or the AcOH/THF/H_2O system,[17] cleavage of the succinic ester moiety occurred, as shown by tests performed on supports **12** and **20**. After exhaustive washings, supports **23** were exploited for the synthesis of the second 3'-5' oligonucleotide fragment, thus completing the desired sequence to give **24**.

Detachment from the solid support and complete deprotection of the synthesized oligomers **a**-**d** were achieved by standard aq. ammonia treatment of supports **21** and **24** for 12 h at 50 °C. Analysis and purification of the crude products were carried out by HPLC on an anion exchange Partisil 10 SAX column. The isolated oligomers were successively desalted on a Sephadex G25 column eluted with H_2O and checked for purity by reverse phase HPLC analysis. For all the synthesized oligomers, similar yields of isolated products were obtained, indicating that both synthetic routes were comparable in terms of efficiency. MALDI-TOF mass spectrometry was employed to characterize modified oligonucleotides **a**-**d**, giving masses in accordance with the expected structures.

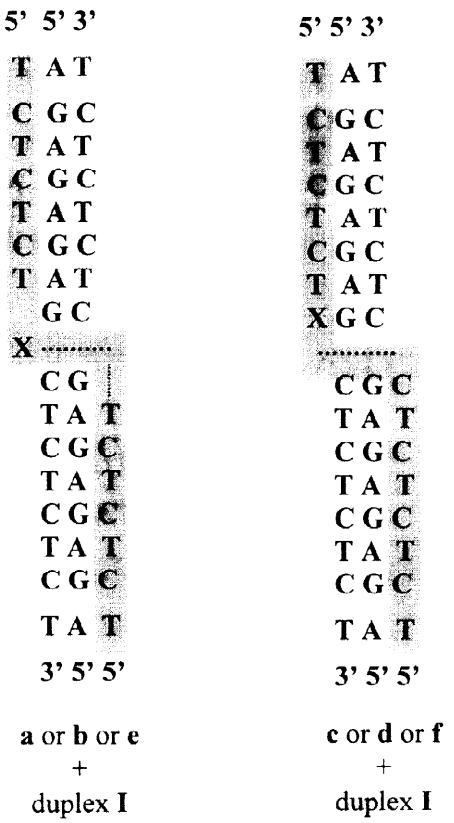
Following the synthetic procedure previously reported[14] and here outlined in Scheme 1, 15-mer **e** and 16-mer **f** (Table 1), containing a 3'-3' phosphodiester bond, were prepared in order to compare their ability to form triplex structures with sequences **a**-**b** and **c**-**d**, respectively.

Thermal denaturation experiments were performed on 1:1 mixtures of 3'-3' oligonucleotides **a**, **b**, **c**, **d**, **e** and **f**, respectively, with 16 bp duplex **I** (Table 2) in a 140 mM KCl, 5 mM NaH_2PO_4, 5 mM $MgCl_2$ solution adjusted to different pH values (5.5, 6.0, 6.6, 7.2). At pH = 7.2 only one S-shaped transition was found in all cases in the T range 10-80 °C, accounting exclusively for the duplex melting. On the other hand, melting profiles in acidic solutions showed a typical biphasic behaviour, with the sigmoids at the lower temperatures due to the triplex-duplex transitions, while the S-shaped curves at higher temperatures were attributed to the expected, pH-insensitive duplex dissociations. The melting temperatures at different acidic pH of the triplexes formed by **a**, **b**, **c** and **d** are shown in Table 2, compared to the data collected, in the same buffer conditions, for all-nucleotide sequences 15-mer **e** and 16-mer **f**. Analysis of these results showed that, in the case of 15-mer **e** targeting 16 bp duplex **I**, the replacement of a nucleosidic unit at the 3'-3' inversion site with a more flexible and less steric demanding molecule - as in oligomers **a** and **b** - caused a certain stabilization of the triple helical complex, at least in the more acidic media, probably owing to the relief in the stress due to the distortion of the triplex structure at the oligopurine-oligopyrimidine junction. On the other hand, we found that the same substitution within the 3'-3' junction in 16-mer **f**, yielding oligomers **c** and **d**, did not favour the triplex formation process, resulting in a detectable loss of affinity towards the target duplex. This could be also ascribed to the existence of both **c** and **d** as pairs of diastereomers, each interacting differently with the double

stranded DNA target.

Even though more flexible, these new 3'-3' linked ODNs did not perform significantly better than the already studied molecules as TFOs; still, they were shown to be able to cooperatively hybridize with target DNA duplexes. Our data suggest that, when no constraints are due to the length of the third strand, the conformation and stereochemistry of the linker play a significative role in the TFO folding at the inversion site, thus measurably influencing the binding properties of the 3'-3' linked third strand. No definitive conclusions can at the moment be drawn about a possible contribution to the binding energy provided by the nucleobases immediately linked or, more generally, adjacent to the 3'-3' junction.

TABLE 2



Tm values (°C)/pH for triplexes with oligonucleotides **a-f**

Sequence	X	pH = 5.5	pH = 6.0	pH = 6.6
a	Gly	33.5	26.7	16.5
b	Cyc	40.5	26.5	16.6
e	Cyt	31.0	22.9	16.7
c	Cly	36.2	26.4	14.8
d	Cyc	27.4	18.2	n.d.
f	Cyt	44.2	36.8	19.8

Gly = glycerine; **Cyc** = cyclohexanetriol; **Cyt** = cytidine; **n.d.** = not detected.

Conclusions.

New solid supports **9**, **12**, **17** and **20**, incorporating suitably functionalized 1,2,3-propanetriol and *cis,cis*-1,3,5-cyclohexanetriol derivatives, were efficiently prepared. They allowed a facile and convenient fully automated synthesis of oligodeoxyribonucleotides containing the cited triols as 3'-3' linkers, having sequences symmetric (**a** and **b**) or asymmetric (**c** and **d**) with respect to the inversion site. In the first case, supports **9** or **17** allowed a simultaneous growing of the oligonucleotide chain from both available hydroxyl functions, thus diminishing by half the number of coupling steps required. On the other hand, supports **12** or **20** gave the

desired asymmetric sequences as a mixture of two diastereomers, due to the prochirality of the glycerine and cyclohexanetriol linkers.

3'-3' modified oligomers were tested in triplex formation experiments as third strands targeting duplexes of the type 5'-$(Pu)_m(Py)_n$-3' in an alternate strand recognition approach. Thermal denaturation data confirmed their ability to cooperatively bind to double stranded DNA fragments. Analysis of the melting temperatures obtained in the same buffers indicated that when these linkers replaced one nucleoside unit at the 3'-3' junction, the affinity towards the target was measurably affected. With sequences symmetric with respect to the inversion site, where also no additional chiral center is introduced with the linker, promising results were found.

In our opinion, the proposed synthetic route is of interest by virtue of the fact that it is in principle easily reproducible with a large number of trifunctional molecules which, once suitably derivatized, can be incorporated in the solid matrix and inserted in oligonucleotide chains as 3'-3' linkers. In addition, the use of the synthesized solid supports can result in a convenient entry to a variety of bis-5'-conjugated ODNs, a class of compounds potentially valuable, and so far not yet investigated, for triplex formation experiments.

Experimental Section

General Methods.

Chromatographies of compounds **6**, 7, **8**, **10**, **11**, **14**, **15**, **16**, **18** and **19** were performed on silica gel columns eluted with increasing amounts of AcOEt in benzene. NMR spectra were recorded on Bruker WM-400 and WM-250 spectrometers. All chemical shifts are expressed in ppm with respect to the residual solvent signal. Tentagel resin was purchased from Rapp Polymere, Tubingen, Germany. The solid support functionalizations with derivatives **8**, **11**, **16** or **19** 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 2'-deoxyribonucleosides as building blocks. In the case of the synthetic oligomer forming duplex **I**, the synthesis and purification were carried out following a standard protocol.[16] Oligomers **e** and **f**, containing a 3'-3' phosphodiester bond, were prepared and purified according to a previously reported procedure.[14] HPLC analyses and purifications were performed on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. Thermal denaturation experiments were carried out on a Cary 1E Varian spectrophotometer equipped with a Haake PG20 thermoprogrammer with detection at $\lambda = 260$ nm.

Functionalization of Tentagel resin: a) preparation of support 9.

0.34 g (3.6 mmol) of 1, 2, 3-propanetriol **5**, dried by repeated coevaporations with anhydrous pyridine and then dissolved in 8 mL of the same solvent, were reacted with 2.5 g (7.2 mmol) of 4,4'-dimethoxytriphenylmethylchloride at r.t. for 12 h. The reaction was quenched by addition of water and the resulting mixture, taken to dryness and redissolved in benzene, was purified on a silica gel column giving 1.63

g (2.34 mmol, 65 % yield) of derivative **6** and 0.35 g (0.90 mmol, 25 %) of derivative **7**. 0.41 g (0.59 mmol) of bistritylated compound **6** were then treated with 0.089 g (0.89 mmol) of succinic anhydride and 0.050 g (0.41 mmol) of 4-dimethylaminopyridine in 2 mL of anhydrous pyridine for 8 h at r.t. The reaction mixture, concentrated under reduced pressure and purified by silica gel chromatography, afforded 0.42 g of succinylated compound **8** (0.53 mmol, 90 %). 0.27 g (0.17 mmol) of **8**, previously coevaporated with anhydrous pyridine, and 0.46 mL of DCCI, 1 M solution in CH_2Cl_2, in 2 mL of anhydrous pyridine, were added to 0.25 g (0.056 mmol of primary ammino groups) of Tentagel resin and the resulting suspension was left overnight at r. t. After washings with CH_2Cl_2, CH_3OH and Et_2O, solid support **9** was dried under reduced pressure. The incorporation yields of derivative **8** were 82 % (0.18 meq/g) 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.

6- yellow foam, R_f (10 % AcOEt/benzene) 0.75; ν_{max}(thin film): 3485 (broad), 1638, 1510, 1385, 1251, 1177, 1032, 831; δ_H (250 MHz, $CDCl_3$): 7.55-6.82 (complex signals, 26H, aromatic protons), 4.07 (m, 1H, C<u>H</u>OH), 3.83 (s, 12H, 4 OCH_3), 3.42 (AB part of an ABX system, *J* 5.2, -10.2, 4H, 2 C<u>H</u>$_2$ODMT), 2.55 (d, *J* 8.0, 1H, OH); δ_C (100.6 MHz, $CDCl_3$): 158.21, 144.71, 135.79, 129.87, 128.07, 127.93, 127.55, 112.86, 85.77, 70.03, 64.08, 54.88; m/z (FAB MS, positive ions): 697 $(M+H)^+$; 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 697.3173. $(C_{45}H_{44}O_7 + H)^+$ requires 697.3165.

8- yellow oil, R_f (30 % AcOEt/benzene) 0.25; ν_{max}(thin film): 3390 (broad), 1735, 1720, 1620, 1505, 1250, 1170, 1012; δ_H (250 MHz, $CDCl_3$): 7.41-6.80 (complex signals, 26H, aromatic protons), 5.34 (t, *J* 4.8, 1H, C<u>H</u>OCOR), 3.82 (s, 12H, 4 OCH_3), 3.37 (d, *J* 4.8, 4H, 2 C<u>H</u>$_2$ODMT), 2.73 (s, 4H, COC<u>H</u>$_2$C<u>H</u>$_2$COOH); δ_C (100.6 MHz, $CDCl_3$): 175.86, 171.59, 158.24, 144.67, 135.78, 129.92, 127.98, 127.62, 126.77, 112.92, 85.70, 72.33, 61.68, 55.02, 29.57, 29.25; m/z (FAB MS, positive ions): 797 $(M+H)^+$; 796 $(M)^+$; 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 797.3331. $(C_{49}H_{48}O_{10} + H)^+$ requires 797.3326.

b) Preparation of support 12.

0.35 g (0.89 mmol) of the monotrityl derivative of glycerine were reacted with 0.16 g of *tert*-butyldimethylsilylchloride (1.1 mmol) and 0.090 g (1.2 mmol) of imidazole in 2 mL of anhydrous DMF at r.t. for 12 h. After purification by silica gel chromatography, 0.44 g (0.87 mmol, 98 % yield) of derivative **10** were recovered. The subsequent treatment of 0.30 g (0.60 mmol) of **10** dissolved in 3 mL of anhydrous pyridine with 0.12 g (1.2 mmol) of succinic anhydride and 0.072 g (0.60 mmol) of 4-dimethylaminopyridine afforded succinylated compound **11**, which, purified on a silica gel column, was recovered in 82 % yields (0.30 g, 0.49 mmol). For the solid support functionalization, 0.30 g of Tentagel resin (0.066 mmol) were reacted with a mixture of 0.25 g (0.42 mmol) of **11** and 0.53 mL of DCCI, 1 M solution in CH_2Cl_2, in 2 mL anhydrous pyridine at r.t. for 24 h. DMT tests performed on dried and weighed samples of resulting support **12** allowed the determination of the incorporation yields of derivative **11** onto the matrix, which were 82 % (0.18 meq/g).

7- yellow foam, R_f (20 % AcOEt/benzene) 0.20; ν_{max}(thin film): 3387 (broad), 1609, 1510, 1252, 1178, 1038, 837; δ_H (250 MHz, $CDCl_3$): 7.49-6.81 (complex signals, 13H, aromatic protons), 3.93-3.83 (submerged signal, m, 1H, C<u>H</u>OH), 3.81 (s, 6H, 2 OCH_3), 3.74-3.58 (complex signal, 2H, C<u>H</u>$_2$OH), 3.33-3.19 (AB part of an ABX system, *J* 6.0, 4.4, 2H, C<u>H</u>$_2$ODMT); δ_C (100.6 MHz, $CDCl_3$): 158.31, 144.47, 135.60, 129.81, 127.88, 127.70, 126.84, 112.98, 86.13, 71.03, 64.66, 64.15, 55.01; m/z (FAB MS, positive ions): 395 $(M+H)^+$, 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 395.1862. $(C_{24}H_{26}O_5 + H)^+$ requires 395.1858.

10- yellow oil, R_f (20 % AcOEt/benzene) 0.40; ν_{max}(thin film): 3491 (broad), 1608, 1510, 1446, 1250, 1176, 1034, 833; δ_H (250 MHz, $CDCl_3$): 7.48-6.79 (complex signals, 13H, aromatic protons), 3.94-3.82 (submerged signal, m, 1H, C<u>H</u>OH), 3.82 (s, 6H, 2 OCH_3), 3.76-3.70 (m, 2H, C<u>H</u>$_2$OTBDMS), 3.30-3.14 (m, 2H, C<u>H</u>$_2$ODMT), 2.52 (d, *J* 9.0, 1H, OH), 0.95 [s, 9H, (C<u>H</u>$_3$)$_3$CSi], 0.10 [s, 6H, (C<u>H</u>$_3$)$_2$Si]; δ_C (100.6 MHz, $CDCl_3$): 158.32, 144.79, 135.98, 129.91, 128.00, 127.64, 126.59, 112.95, 85.88, 71.63, 70.99, 63.98, 55.03, 25.71, 18.15, -5.40; m/z (FAB MS, positive ions): 508 $(M)^+$, 377 (M - *t*-$BuMe_2SiO)^+$, 303 $(DMT)^+$; HRMS (FAB): $(M)^+$ found 508.2649. $C_{30}H_{40}O_5Si$ requires 508.2645.

11- yellow oil, R_f (30 % AcOEt/benzene) 0.20; ν_{max}(thin film): 3415 (broad), 1737, 1719, 1610, 1510, 1440, 1251, 1171, 1028; δ_H (250 MHz, $CDCl_3$): 7.49-6.80 (complex signals, 13H, aromatic protons), 5.17 (m, 1H, C<u>H</u>OCOR), 3.79 (s, 6H, 2 OCH_3), 3.78 (m, 2H, C<u>H</u>$_2$OTBDMS), 3.26 (m, 2H, C<u>H</u>$_2$ODMT), 2.70 (s, 4H, COC<u>H</u>$_2$C<u>H</u>$_2$COOH), 0.83 [s, 9H, (C<u>H</u>$_3$)$_3$CSi], 0.024 and 0.013 [s's, 3H each, (C<u>H</u>$_3$)$_2$Si]; δ_C (100.6 MHz, $CDCl_3$): 176.64; 171.59, 158.24, 144.65, 135.86, 129.85, 127.95, 127.57, 126.52, 112.88, 86.10, 73.87, 61.85, 61.58, 54.97, 29.09, 25.56, 17.94, -5.62; m/z (FAB MS, positive ions): 608 $(M)^+$, 303 $(DMT)^+$; HRMS (FAB): $(M)^+$ found 608.2815. $C_{34}H_{44}O_8Si$ requires 608.2805.

c) Preparation of support 17.

Analogously to the procedure described above for the functionalization of Tentagel with **8**, reaction of **13** (0.40 g, 2.38 mmol) with 1.6 g (4.76 mmol) of DMTCl in 8 mL of anhydrous pyridine led to bistritylated derivative **14** (0.83 g, 1.12 mmol, 47 % yield) and monotritylated derivative **15** (0.28 g, 0.64 mmol, 27 % yield), after silica gel column. 0.40 g (0.54 mmol) of cyclohexanetriol derivative **14** were treated with succinic anhydride (85 mg, 0.85 meq) and 4-dimethylaminopyridine (45 mg, 0.37 meq) in 2 mL of anhydrous pyridine overnight at r. t. giving, after silica gel chromatography, 0.37 g of pure succinylated compound **16** (0.44 mmol, 82 % yield). 0.39 g of Tentagel resin (0.086 mmol of reactive primary amino groups) were then left in contact for 24 h at r. t. with a 2 mL anhydrous pyridine solution of **16** (0.30 g, 0.36 mmol), previously coevaporated with anhydrous pyridine, and 0.52 mL of DCCI, 1 M solution in CH_2Cl_2. After exhaustive washings with CH_2Cl_2, CH_3OH and Et_2O, support **17** was dried under reduced pressure. Functionalization of support **17**, measured by DMT test, was 0.16 meq/g (73 % yield).

14- yellow oil, R_f (20 % AcOEt/benzene) 0.70; ν_{max}(thin film): 3464 (broad), 1608, 1505, 1445, 1245, 1176, 1045, 834; δ_H (250 MHz, $CDCl_3$): 7.40-6.70 (complex signals, 26H, aromatic protons), 3.67 (s, 12H, 4 OCH_3),

3.10 (apparent triplet, *J* 10.1, 9.8, 2H, 2 CHODMT), 2.83 (apparent triplet, *J* 10.5, 9.6, 1H, CHOH), 1.67-1.03 (overlapped signals, 6H, 3 CH_2); δ_C (100.6 MHz, $CDCl_3$): 158.13, 145.97, 136.98, 130.03, 128.12, 127.42, 126.43, 112.71, 85.86, 67.56, 65.56, 54.89, 42.32, 41.08; m/z (FAB MS, positive ions): 737 $(M+H)^+$, 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 737.3482. $(C_{48}H_{48}O_7 + H)^+$ requires 737.3478.

16- yellow oil, R_f (10 % AcOEt/benzene) 0.35; ν_{max}(thin film): 3427 (broad), 1732, 1720, 1609, 1508, 1442, 1248, 1173, 1040; δ_H (250 MHz, $CDCl_3$): 7.46-6.75 (complex signals, 26H, aromatic protons), 4.02 (m, 1H, CHOCOR), 3.71 and 3.69 (2 s's, 6 H each, 4 OCH_3), 3.18 (m, 2H, 2 CHODMT), 2.52 and 2.48 (2 t's, *J* 7.2 and 7.0, 2H each, $COCH_2CH_2COOH$), 1.70-1.10 (complex signals, 6H, 3 CH_2); m/z (FAB MS, positive ions): 837 $(M+H)^+$, 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 837.3640. $(C_{52}H_{52}O_{10} + H)^+$ requires 837.3638.

d) Preparation of support 20.

Treatment of 0.28 g (0.64 mmol) of the monotrityl derivative of cyclohexanetriol **15** with *tert*-butyldimethylsilylchloride (0.12 g, 0.77 mmol) and 0.065 g (0.96 mmol) of imidazole in 1.5 mL of anhydrous DMF at r.t. for 12 h allowed diprotected triol **18** to be recovered in 92 % yield (0.33 g, 0.59 mmol) after silica gel column. Succinylated derivative **19** was then obtained in 90 % yield, after purification (0.345 g, 0.53 mmol), reacting **18** (0.33 g, 0.59 mmol) with succinic anhydride (0.12 g, 1.2 mmol) and 0.072 g (0.60 mmol) of 4-dimethylaminopyridine. Successively 0.23 g of Tentagel support (0.050 mmol) were left in contact with a 1.5 mL pyridine solution of 0.10 g (0.30 mmol) of **19** and 0.4 mL of DCCI, 1 M solution in CH_2Cl_2, at r.t. for 24 h. Functionalization of support **20** was 0.15 meq/g, accounting for a 68 % incorporation yield.

15- yellow oil, R_f (20 % AcOEt/benzene) 0.50; ν_{max}(thin film): 3390 (broad), 1607, 1509, 1459, 1251, 1177, 1031, 829; δ_H (250 MHz, $CDCl_3$): 7.52-6.82 (complex signals, 13H, aromatic protons), 3.74 (s, 6H, 2 OCH_3), 3.55-3.32 (overlapped signals, 3H, 2 CHOH and CHODMT protons), 1.62-1.20 (overlapped signals, 6H, 3 CH_2); δ_C (100.6 MHz, $CDCl_3$): 158.51, 145.79, 136.94, 130.20, 128.31, 127.69, 126.75, 113.05, 86.56, 67.85, 65.99, 55.14, 43.11, 41.67; m/z (FAB MS, positive ions): 435 $(M+H)^+$, 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 435.2180. $(C_{27}H_{30}O_5 + H)^+$ requires 435.2171.

18- yellow oil, R_f (5 % AcOEt/benzene) 0.65; ν_{max}(thin film): 3382 (broad), 1607, 1508, 1463, 1251, 1178, 1037, 835; δ_H (250 MHz, $CDCl_3$): 7.55-6.82 (complex signals, 13H, aromatic protons), 3.80 (s, 6H, 2 OCH_3), 3.50-3.18 (overlapped signals, 3H, CHOH and 2 CHOR protons), 2.07-1.65 (complex signals, 3H, equatorial protons of CH_2 groups), 1.48-1.17 (complex signals, 3H, axial protons of CH_2 groups), 0.84 [s, 9H, $(CH_3)_3CSi$], -0.046 and -0.065 [2 s's, 3H each, $(CH_3)_2Si$]; δ_C (100.6 MHz, $CDCl_3$): 158.28, 145.93, 137.12, 130.47, 128.34, 127.60, 127.53, 113.05, 86.06, 67.51, 65.91, 65.83, 54.98, 43.94, 42.67, 42.56, 25.60, 17.91, -5.03; m/z (FAB MS, positive ions): 549 $(M+H)^+$, 493 (M-isobutylene)$^+$, 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 549.3038. $(C_{33}H_{44}O_5Si + H)^+$ requires 549.3036.

19- yellow oil, R_f (50 % AcOEt/benzene) 0.55; ν_{max}(thin film): 3395 (broad), 1737, 1721, 1610, 1509, 1460, 1250, 1177, 1030; δ_H (250 MHz, $CDCl_3$): 7.60-6.87 (complex signals, 13H, aromatic protons), 4.54 (m, 1H,

CHOCOR), 3.86 (s, 6H, 2 OCH_3), 3.50 and 3.31 (m's, 1H each, CHODMT and CHOTBDMS), 2.67 (m, 4H, $COCH_2CH_2COOH$), 2.24-1.80 (complex signals, 3H, equatorial protons of CH_2 groups), 1.50 -1.21 (complex signals, 3H, axial protons of CH_2 groups), 0.86 [s, 9H, $(CH_3)_3CSi$], -0.017 and -0.039 [2 s's, 3H each, $(CH_3)_2Si$]; δ_C (100.6 MHz, $CDCl_3$): 176.52, 171.16, 158.41, 145.91, 136.97, 130.14, 128.1, 127.59, 126.60, 112.98, 86.23, 68.30, 67.25, 65.99, 55.03, 42.98, 40.52, 38.64, 29.15, 28.87, 25.64, 17.86, -5.14; m/z (FAB MS, positive ions): m/z 649 $(M+H)^+$, 303 $(DMT)^+$; HRMS (FAB): $(M+H)^+$ found 649.3202. $(C_{37}H_{48}O_8Si + H)^+$ requires 649.3197.

Synthesis and characterization of the 3'-3' linked adenosine dinucleotide phosphate g and h.

Dimers **g** and **h** were synthesized using both the symmetric and the asymmetric approaches, obtaining in all cases very similar yields of the final products. In the first case, 50 mg of support **9** or **17** have been subjected to a single coupling cycle using 2'-deoxyadenosine 3'-phosphoramidite in 45 mg/mL concentration as building block, following a standard automated protocol.[16] In the second case, 50 mg of support **12** or **20** were in use; addition of the first 2'-deoxyadenosine 3'-phosphate residue was performed first, followed by final DMT removal and benzoylation of the 5'-OH end by treatment with 0.5 mL of benzoyl chloride in 0.5 mL pyridine for 30 min at r. t. Subsequently, TBDMS removal, by addition of $Et_3N{\cdot}3HF$ (80 μL, 0.41 meq) in 450 μL of anhydrous THF at r.t. for 18 h, allowed deprotection of the remaining hydroxyl function; this was then reacted with the second 2'-deoxyadenosine unit, followed by final DMT removal.

Deprotection and detachment of the synthesized dimers from the solid supports were obtained by treatment with conc. aq. ammonia (overnight, 50 °C). The supernatant was filtered and the support washed with water. The combined filtrate and washings were concentrated *in vacuo*, redissolved in water, analyzed and purified by HPLC on a Partisil 10 SAX column (Whatman, 4.6 x 250 mm, 7 μm) eluted with a linear gradient from 0 to 7 % in 30 min of eluent B in A (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), flow 0.8 mL/min. The isolated dimers, having the following retention times: **g** = 12.48 min; **h** = 13.14 min, were collected and successively desalted by HPLC on an analytical RP18 Lichrosorb column (Merck, 4.6 x 250 mm, 5 μm) eluted with 3 % CH_3CN in H_2O. The peaks showing the following retention times: 5.02 min for **g** and 5.75 min for **h**, accounting for more than 99 % of the crude profiles, were identified as the desired dimers by ^1H- and ^{31}P-NMR spectroscopy and FAB MS data.

g - δ_H (400 MHz, D_2O): 8.15 (s, 2H, 2 H-2), 7.96 (s, 2H, 2 H-8), 6.29 (dd, 2H, 2 H-1'), 4.91 (m, 2H, 2 H-3'), 4.33 (m, 2H, 2 H-4'), 4.04 (m, 5H, 2 CH_2OR and CHOH of the glycerine unit), 3.78 (m, 4H, 2 H_2-5'), 2.69 (m, 4H, 2 H-2'); δ_P (161.98 MHz, D_2O, 85 % H_3PO_4 as external standard): 2.95; m/z (FAB MS, negative ions): 717 $(M-H)^-$; HRMS (FAB): $(M-H)^-$ found 717.1558. $(C_{23}H_{32}O_{13}N_{10}P_2 - H)^-$ requires 717.1547.

h - δ_H (400 MHz, D_2O): 8.42 e 8.41 (2 s's, 1H each, 2 H-2), 8.32 and 8.31 (2 s's, 1H each, 2 H-8), 6.52-6.44 (2 dd's, 2H, 2 H-1'), 5.03 (m, 2H, 2 H-3'), 4.43 (m, 2H, 2 H-4'), 4.27 (m, 2H, 2 CH of the cyclohexanetriol unit adjacent to the phosphate unit), 3.90 (m, 4H, 2 H_2-5'), 3.80 (m, 1H, CHOH of the cyclohexanetriol unit), 2.97-

2.80 (m, 4H, 2 H_2-2'), 2.59 (m, 1H, H_{eq} of CH_2 of cyclohexanetriol unit adjacent to phosphate groups), 2.48 (m, 2H, 2 H_{eq} of two CH_2 of cyclohexanetriol adjacent to CHOH), 1.68 (q, 1H, H_{ax} of CH_2 of cyclohexanetriol unit adjacent to phosphate groups), 1.51 (q, 2H, 2 H_{ax} of two CH_2 of cyclohexanetriol adjacent to CHOH); δ_P (161.98 MHz, D_2O, 85 % H_3PO_4 as external standard): 2.13; m/z (FAB MS, negative ions): 757 $(M-H)^-$; HRMS (FAB): $(M-H)^-$ found 757.1879. ($C_{26}H_{36}O_{13}N_{10}P_2$ - H)$^-$ requires 757.1860.

Chain assembly of symmetric oligomers a and b.

For the preparation of ODNs **a** and **b**, 50 mg (0.0065 meq) of functionalized support, **9** or **17**, were used in the standard automated synthesis of the sequence $^{5'}$TCTCTCT$^{3'}$, with final DMT removal. Chain elongation on both 5'-ends of the supports was carried out using longer coupling cycles (8 min) and higher phosphoramidites concentrations (45 mg/mL) than in standard automated procedures,[16] yielding the desired symmetric sequences $^{5'}$TCTCTCT$^{3'}$-linker-$^{3'}$TCTCTCT$^{5'}$ (**21**, Scheme 3).

Chain assembly of asymmetric oligomers c and d.

The synthesis of sequences **c** and **d**, asymmetric with respect to the 3'-3' linker, was typically carried out starting from 50 mg (0.0065 meq) of derivatized support **12** or **20** respectively. Once the first domain of the ODN chain was assembled following standard automated methods,[16] the final DMT group was removed and a prolonged (30 min, r.t.) treatment with 0.5 mL of benzoyl chloride in pyridine (0.5 mL) was performed to block the 5'-end, leading to **22** (Scheme 3). The removal of TBDMS protecting group was achieved by $Et_3N{\cdot}3HF$ (80 μL, 0.41 meq) in 450 μL of anhydrous THF at r.t. for 18 h. Resulting supports **23** were successively coupled with the required 3'-phosphoramidite derivatives to complete the desired sequence following standard automated procedures,[16] including final DMT removal, to give **24**.

Deprotection, purification and characterization of oligomers a-d.

Deprotection and detachment of the synthesized oligomers from solid supports **21** and **24** were achieved by an overnight treatment with conc. aq. ammonia at 50 °C. The supernatant was filtered and the support washed with water. The combined filtrate and washings were concentrated *in vacuo*, redissolved in water, analyzed and purified by HPLC on a Partisil 10 SAX column (Whatman, 4.6 x 250 mm, 7 μm) eluted with linear gradients of KH_2PO_4 (20 % CH_3CN, pH = 7.0) from 1 to 350 mM in 30 min, flow 0.8 mL/min. The isolated oligomers, having the following retention times: **a** = 20.59 min; **b** = 20.66 min; **c** = 23.38 min; **d** = 21.36 min, were collected and successively desalted by gel filtration on a Sephadex G25 column eluted with H_2O.

By HPLC analysis on a Partisphere Whatman RP18 analytical column (125 x 4.0 mm, 5 μm), the isolated 3'-3' oligomers resulted to be more than 98 % pure. Using a linear gradient (from 5 to 50 % in 30 min) of CH_3CN in 0.1 M aq. triethylammonium bicarbonate buffer, pH = 7.0 (flow 0.8 mL/min, detection at λ = 260 nm), they showed the following retention times: **a** = 13.11 min; **b** = 14.21 min; **c** = 14.68 min; **d** = 15.03 min.

The synthesized compounds were characterized by MALDI-TOF MS: oligonucleotide **a**, mass calculated $[M-H]^-$ = 4098; mass observed 4099; oligonucleotide **b**, mass calculated $[M-H]^-$ = 4138; mass observed 4138; oligonucleotide **c**, mass calculated $[M-H]^-$ = 4387; mass observed 4389; oligonucleotide **d**, mass calculated $[M-H]^-$ = 4427; mass observed 4428.

Thermal denaturation experiments.

The concentration of the synthesized ODNs was determined spectrophotometrically at λ = 260 nm and at 85 °C, using the following molar extinction coefficients for each base:[18] 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 the melting experiments, 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 resulting solutions were then allowed to heat at 80 °C for 15 min, then slowly cooled 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 temperature, increased at a rate of 0.5 °C/min, typically in the range 10-80 °C. The melting temperatures, reported in Table 2, were determined as the maxima of the first derivative of absorbance *vs.* temperature plots.

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References and Notes.

• indicates Hoogsteen-type interactions while x stands for Watson-Crick interactions.

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