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Synthesis and Properties of Oligo-2′-deoxyribonucleotides Containing Internucleotidic Phosphoramidate Linkages Modified with Pendant Groups Ending with either Two Amino or Two Hydroxyl Functions

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Abstract—Single and multiple incorporations of stereochemically pure modified dinucleoside-phosphoramidates involving substituent groups ending with bis-hydroxyethyl and bis-aminoethyl groups have been performed into pyrimidic triple helix-forming oligo-2′-deoxyribonucleotides designed to bind parallel to the purine strand of the DNA target. The ability of these modified oligo-2′-deoxyribonucleotides to form triple helices has been studied by UV-melting curve analyses, and circular dichroism. Only the oligonucleotides involving modified phosphate groups with the Rp configuration formed more stable triple helices than did the parent phosphodiester sequences. Incorporating the modifications into the third oligonucleotide strands has little effect on the structure of the triplexes. At pH 7, the incorporation of two, three or four modified phosphate groups into the third strands stabilizes the triplexes, as compared to the unmodified oligonucleotide. Stronger stabilization was observed with compounds containing linkers ending with amino functions. Stability increases with the number of modifications without being fully additive. This might be due to the different environments of the phosphate groups inside the sequence.

Introduction

Numerous applications have been found for oligonucleotides, because of their hybridization properties, in various areas such as biotechnology and diagnostics. Their potential use as therapeutics in regulating gene expression following the antisense, antigene, ribozyme and aptamers strategies has stimulated a great deal of research work.[1–6] The antigene strategy relies on the formation of triple-helical nucleic acid structures involving synthetic oligonucleotides that recognize short oligopyrimidine·oligopurine runs in double-stranded DNA via Hoogsteen or reverse Hoogsteen hydrogen bond formation with purine bases in the major groove. The triple helix formation with short third strand oligonucleotides was first reported for pyrimidine oligonucleotides,[7,8] then for oligonucleotides containing G and T,[9] G and A,[10,11] and T, C and G.[12] The recognition of oligopurine runs by the above reported oligonucleotides involves the formation of T.AxT, C.GxC+, C.GxG and T.AxA base triplets. A pyrimidine third strand binds parallel to the purine strand of the double-stranded target to form T·AxT and C·GxC$^+$ isomorphous triplets via Hoosgsteen hydrogen bonding (where C$^+$ indicates protonated cytosine and the symbols · and x stand for Watson–Crick and Hoogsteen hydrogen bonds, respectively). The binding stability of the C/T-containing pyrimidine third strand is pH sensitive because its cytosine bases can only form two Hoogsteen bonds when protonated, rendering the targeting of C·G stretches difficult due to the charge-charge repulsion between contiguous protonated cytosines. A third purine strand binds in an anti-parallel orientation forming C·GxG and T·AxA base triplets via reverse Hoogsteen hydrogen bonding. G- and T-containing oligonucleotides can also form triplexes whose third strand orientation is sequence dependent.

The stability of triple-stranded RNA and DNA could be increased when the electrostatic repulsion among the polyanionic single strands was reduced by partial or complete replacement of the negatively charged phosphodiester by either neutral or positively charged groups. Along these lines, several families of modified

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oligonucleotides have been developed that can be separated into the following two broad structural classes: those that retain the nucleoside and phosphorus backbone of DNA and incorporate a pendant group at the phosphorus atom either neutral, as in the case of methylphosphonate, phosphotriester, or involving amino function(s) that can be protonated at physiological pH via phosphonates or phosphoramidates linkages,[13–27] and those that replace the phosphate internucleotide linkage with other chemical units such as formacetal, MMI, guanidinium or methylthiourea.[28–36] More important modifications of the oligonucleotide backbone involving the replacement of sugar and phosphate units have been reported such as in the PNA[37] and the pyrrolidine-amide oligonucleotide mimic.[38] Among these different families of modified oligonucleotides, we have focused our attention on those that incorporate a pendant group at the phosphorus atom. The replacement of one non-bridging oxygen atom by any substituent induces the formation of a chiral center at the phosphorus atom which leads to a mixture of diastereoisomers. It has been shown that the stability of complexes formed between modified oligonucleotides and their complementary target sequences depends on the stereochemistry of the P-chiral internucleoside linkages.[15,16,22,24] In addition, steric hindrance of the substituent at a phosphorus atom also has a negative effect on the stability of the complexes.

We report here single and multiple incorporations of a series of modified dinucleoside-phosphoramidates involving substituent groups, ending with either two hydroxyl or two amino functions, into 22-mer and 17-mer pyrimidic TFOs, respectively. These modified TFOs are designed to bind in a parallel orientation relative to the purine strand of the DNA target. For triple helix formation we have chosen, as a model, a 24-bp long target involving a 22-bp oligopyrimidine·oligopurine sequence, present on the exon 11 of BRCA1 gene. This sequence involves a run of five thymines but is devoid of consecutive cytosines in order to avoid the problem of charge repulsion between positively charged cytosines. Both strands of the target sequence were joined at their extremities with hexaethylene glycol linkers in order to enhance the thermal stability of the double-stranded DNA target. The ability of these modified oligonucleotides to form triple helices has been studied using UV-melting curve analysis and circular dicroism spectroscopy.

Results and Discussion

Experimental design

A survey of the literature shows that among the various methods used to increase the affinity of oligonucleotides for their double-stranded target is the possibility of introducing an amino function, that can be protonated, at physiological pH, so as to reduce the electrostatic repulsion between the third strand oligonucleotide and the DNA target. Another approach consists in introducing groups that are able to form additional hydrogen bonding with the target. Binding properties of the reported modified ODNs depend on many parameters: the number of positively charged groups, their location along the sequence, the structure and length of the linkers used to connect the positively charged pendant groups to the ODN as well as the stereochemistry when the substitution is performed at the phosphorus atom level. We chose to introduce at internucleotidic positions via phosphoramidate linkages, pendant groups involving a tertiary amino group and two other groups, either two amino or two hydroxyl functions (Fig. 1). In addition, we chose to change the length of the linker between the phosphoramidate linkage and the tertiary amino function by using either ethyl or propyl groups. The synthesis of the linkers and the modified oligonucleotides was performed as depicted in Schemes 1 and 2, respectively. As this modification introduces chirality at the phosphorus atom, stereochimically pure Rp and Sp dinucleoside-phosphoramidates with either of the above described linkers were prepared and incorporated into the oligonucleotides for evaluation of their effect on binding stability. Identification of the stereochemistry of the modified internucleotidic linkage confirmed that only the oligonucleotides with the Rp configuration at the modified phosphorus form slightly more stable triplexes than do the unmodified oligonucleotides, used as reference. Results also indicated that slightly more stable triplexes were obtained with a bis-hydroxylated linker involving a propyl chain between the phosphorus atom and the tertiary amino function, as well as with a bis-aminated linker involving the ethyl group. Multiple incorporations (2, 3 and 4) of dinucleoside-phosphoramidates involving the selected linkers with the Rp configuration, have been performed into 17-mer oligonucleotides. As a target for triple helix formation, we chose a 22-bp oligopyrimidine·oligopurine sequence which is depicted in Figure 2 as part of a synthetic 24-bp long duplex bridged by two hexaethylene glycol linkers.[39,40] Triplex-forming oligonucleotides (TFOs) specific to this oligopurine run are aligned below it.

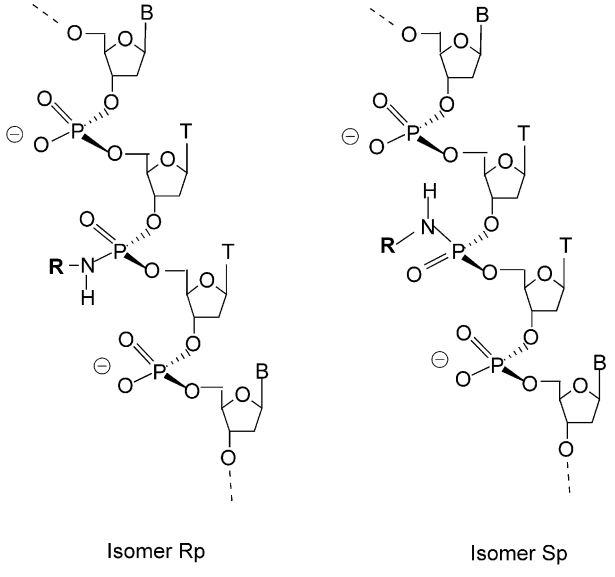
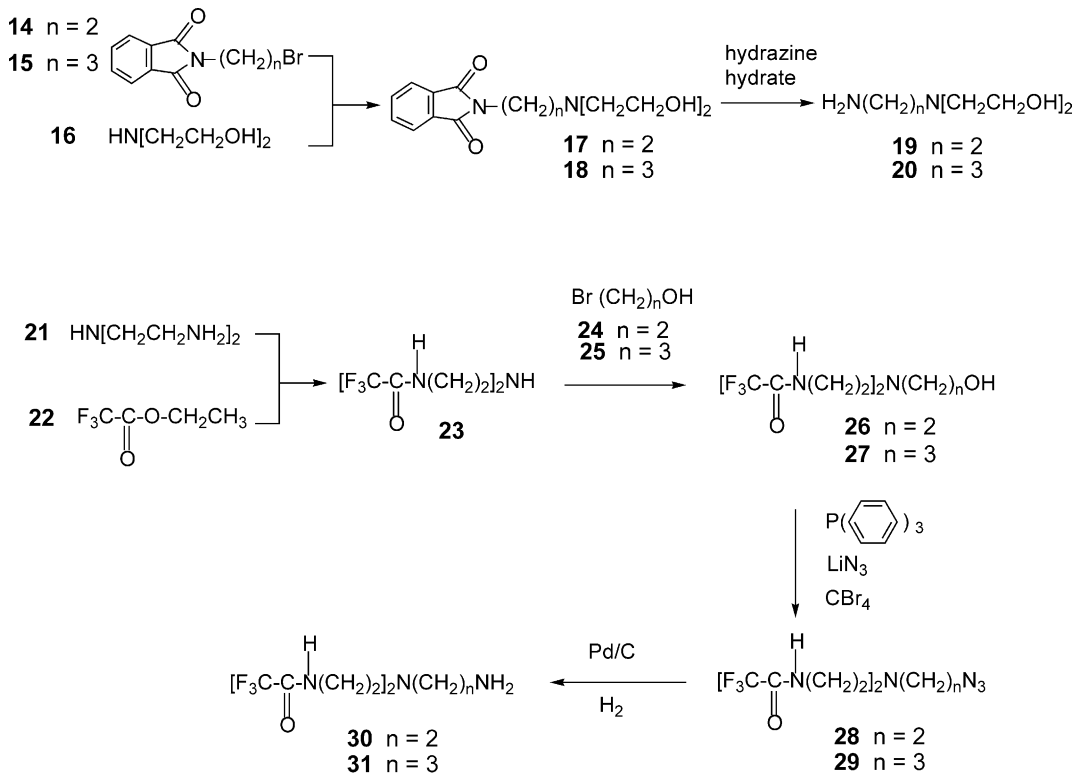


Figure 1. Structures of the modified internucleotidic linkages.



Scheme 1. Synthesis of substituent groups **19**, **20**, **30** and **31** used for the internucleotidic modifications.

Preparation of the linkers

The bis-hydroxylated linkers **19** and **20** were obtained as described in Scheme 1 by reaction of *N*-(2-bromoethyl)phthalimide **14** or *N*-(3-bromopropyl)phthalimide **15** with diethanolamine (9 equiv) **16** followed by treatment with hydrazine hydrate. The bis-aminated linkers **30** and **31** were obtained following a four-step procedure as described in Scheme 1. First, both amino functions of diethylenetriamine **21** were trifluoroacetylated by reaction with ethyltrifluoro-acetate **22** to give compound **23**.[41] The latter was then reacted in acetonitrile with one equivalent of either bromoethanol **24** or bromopropanol **25** in the presence of excess triethylamine (1.5 equiv) at 50 °C for 24 h. The obtained hydroxylated linkers **26** and **27** were transformed separately into azido derivatives **28** and **29** by reaction with triphenylphosphine (1.5 equiv) and lithium azide (3 equiv), in the presence of carbon tetrabromide (1.5 equiv). Aminated linkers **30** and **31** were obtained by hydrogenation in the presence of 10% Pd on activated charcoal.

Synthesis of modified dinucleosides 37a, 37b, 38a, 38b, 43a, 43b, 44a and 44b and preparation of their phosphoramidite derivatives 39a, 39b, 40a, 40b, 45a, 45b, 46a and 46b

Preparation of modified dinucleosides involving protected bis-hydroxylated linkers 33a–38b. Starting from each pure diastereoisomer **32a** and **32b** of the fully protected dinucleoside-3′-H-phosphonate **32**, obtained following a procedure adapted from the literature,[42] these compounds were synthesized following a three-step procedure as described in Scheme 2. First, dinucleoside-3′-H-phosphonates **32a** and **32b** were separately reacted with an excess of hydroxylated linkers **19** ($n=2$) and **20** ($n=3$) in the presence of CCl_4 and pyridine to give two pairs of dinucleosides, **33a**, **33b** and **34a**, **34b**, involving a pendant group ending with hydroxyl functions. These hydroxyl functions were then protected by benzoylation to give two pairs of fully protected dinucleosides **35a**, **35b** and **36a**, **36b**, respectively. The latter were desilylated by treatment with tetrabutylammonium fluoride to afford two pairs of dinucleosides **37a**, **37b** and **38a**, **38b**.

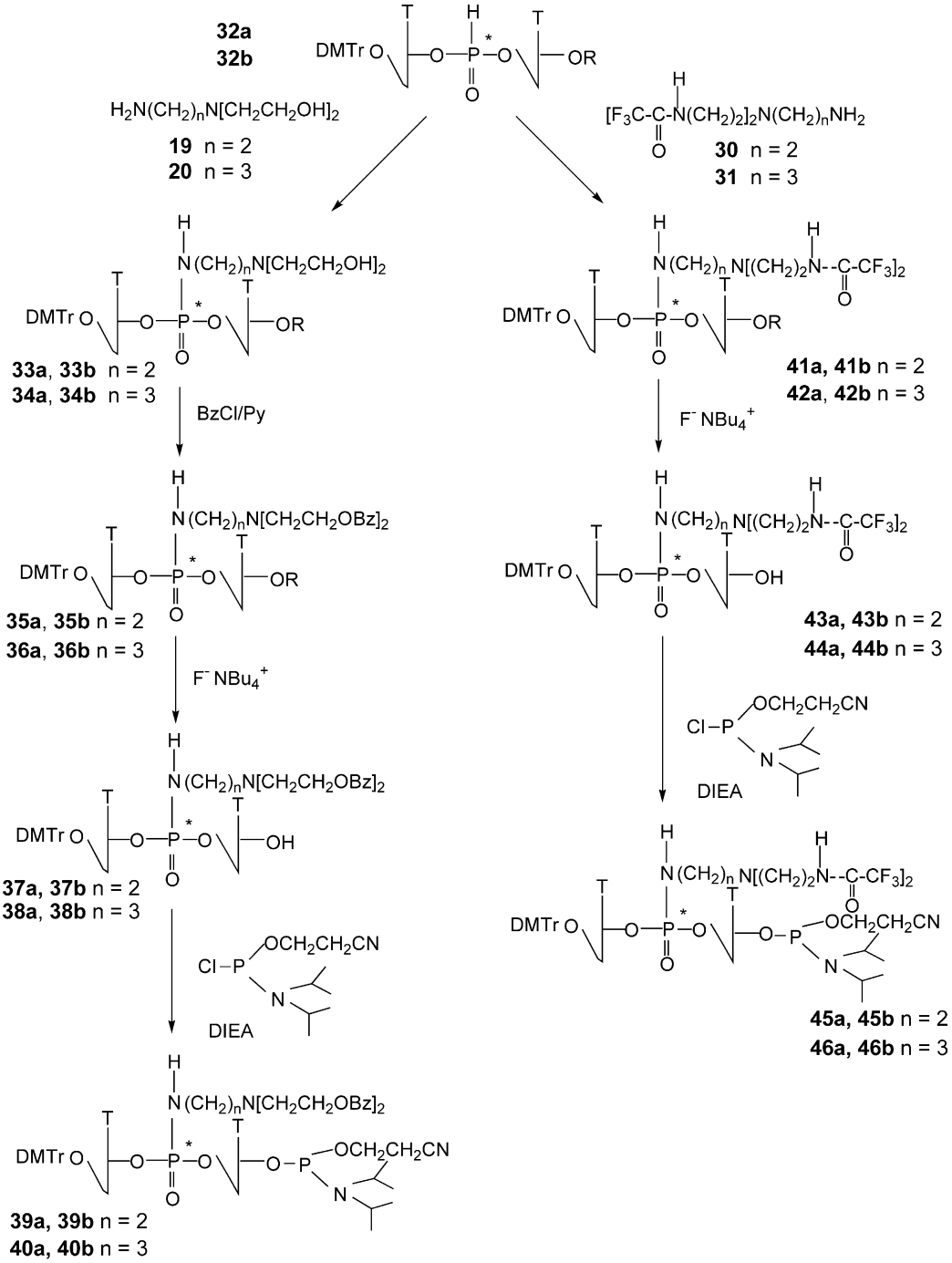
Preparation of modified dinucleosides involving protected bis-aminated linkers 43a, 43b, 44a and 44b. Starting from each pure diastereoisomer **32a** and **32b** of the fully protected dinucleoside H-phosphonate **32** (see above), these compounds were obtained following a two-step procedure as described in Scheme 2. First, dinucleoside-3′-H-phosphonates **32a** and **32b** were separately reacted with an excess of aminated linkers **30** ($n=2$) and **31** ($n=3$) in the presence of CCl_4 and pyridine to give two pairs **41a**, **41b** and **42a**, **42b** of the dinucleosides involving a pendant group ending with two protected amino functions. The latter were desilylated by treatment with tetrabutylammonium fluoride to give two pairs **43a**, **43b** and **44a**, **44b**.

Preparation of phosphoramidite derivatives 39a–40b and 45a–46b of modified dinucleosides 37a–38b and 43a–44b. Starting from dinucleosides **37a**–**38b** and **43a**–**44b**, the phosphoramidite derivatives were obtained following a procedure adapted from the literature.[43] In the case of compounds **45a**–**46b**, the purification step on silica gel

column was omitted in order to prevent partial loss of the trifluoroacetyl groups. These modified phosphoramidite derivatives were purified by precipitation in cold hexane.

Synthesis of oligonucleotides bearing one 2a, 2b, 3a, 3b, 4a, 4b, 5a, 5b, or several 7–12 appended linker(s) ending with either two hydroxyl or two amino functions at the internucleotidic position

Oligo-2′-deoxyribonucleotides containing either a single **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a**, **5b** or multiple incorporations **7**–**12** of the modified dinucleosides were assembled via phosphoramidite chemistry on a CPG support at a 1-μmol scale. For the coupling of modified dinucleoside-3′-phosphoramidites **39a**, **39b**, **40a**, **40b**, **45a**, **45b**, **46a** or **46b**, described above, the concentration of amidites was 1.5 times that of the unmodified nucleoside-3′- phosphoramidites and the coupling and detritylation (after incorporation of the modified dimer) times were increased by 20%. Purification was performed by ion exchange chromatography. After the desalting step, the purity of the modified oligonucleotides was verified by both ion-exchange and reversed-phase chromatography analyses. The results of ion exchange analyses performed at pH 7 clearly show that the modified oligonucleotides with pendant groups ending with hydroxyl



Scheme 2. Synthesis of modified dinucleoside-3′-phosphoramidites **39a**–**40b** and **45a**–**46b**. R = *tert*-butyldimethylsilyl, Bz = benzoyl.

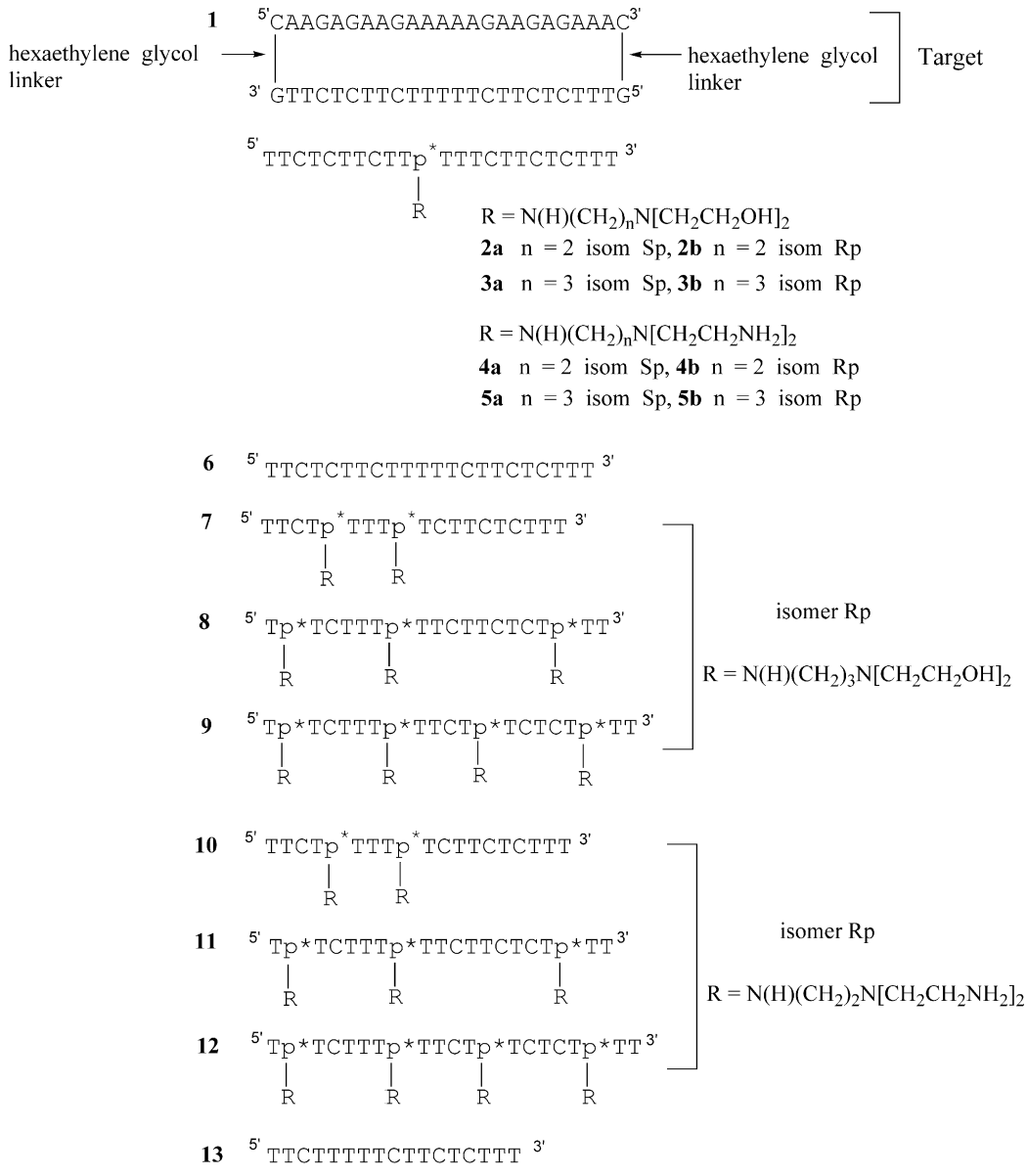


Figure 2. Chemical structures of triple-helical complexes. The sequences of the circularized 24-bp long double-stranded target containing the 22-bp long oligopyrimidine·oligopurine sequence and the third strands are depicted.

functions are more retained that those with pendant groups ending with amino groups (see Experimental for retention times). These results are consistent with the possibility of protonation of the amino functions at pH 7. Oligonucleotides **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a** and **5b** were characterized by nuclease degradation analysis which confirmed the presence and the number of the modified dinucleosides. Electrospray Mass Spectroscopy Analysis of oligonucleotides **2a** to **5b** and **7**–**12** confirmed their mass (Table 1). The purity of the modified oligonucleotides was also confirmed by polyacrylamide gel electrophoresis analyses in denaturing conditions (Figs 3 and 4).

Determination of the configuration of isomers 2a, 2b, 3a, 3b, 4a, 4b, 5a and 5b of modified 22-mer oligonucleotides

This was achieved as previously reported.[44] The fully protected dinucleoside-H-phosphonate **32** was obtained following a procedure adapted from the literature.[42] Dinucleoside isomers **32a** and **32b** were purified and separated by silica gel chromatography. A part of each pure dinucleoside-H-phosphonate isomer **32a** and **32b** was separately transformed (with retention of configuration at the phosphorus center) into pure dinucleoside-phosphorothioate by treatment with S_8 in a pyridine/CS_2 mixture.[42] After deprotection and purification by reversed-phase chromatography, aliquots of each pure dinucleoside-phosphorothioate isomer was treated separately with P1 endonuclease and snake venom phosphodiesterase (SVP) and then with alkaline phosphatase (AP). Treatment with P1 + AP led to the full degradation of the Sp isomer into dT while the Rp isomer remained unchanged. On the contrary, treatment with SVP + AP led to full degradation of the Rp isomer into dT while the Sp isomer remained unchanged.[45] Our results showed that only the dinucleoside-phosphorothioate from dinucleoside-H-phosphonate **32a** was cleaved by P1 + AP treatment, while only the dinucleo-

side-phosphorothioate from dinucleoside-H-phosphonate **32b** was cleaved by SVP + AP treatment. Since sulfurization of H-phosphonate is a stereoselective process, according to the Cahn-Ingold-Prelog rules, the dinucleoside-H-phosphonate **32a** possesses the Rp configuration and the dinucleoside-H-phosphonate **32b** the Sp configuration. Since the conversion of dinucleoside-H-phosphonates **32a** and **32b** to dinucleosides **33a**–**34b** and **41a**–**42b** occurs with inversion of configuration,[46] we were able to conclude that oligonucleotides **2a**, **3a**, **4a** and **5a** exhibit the Sp configuration and oligonucleotides **2b**, **3b**, **4b**, and **5b** the Rp configuration.

Binding analysis: T_m measurements by UV absorption

In a first step oligonucleotides bearing a single incorporation of modified internucleotide linkages, **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a**, **5b**, were compared with unmodified oligonucleotide **6**. In the presence of 5 mM $MgCl_2$ and 140 mM KCl in 10 mM sodium cacodylate buffer, pH 7, two transitions were observed for all the oligonucleotides studied reflecting the dissociation of the TFO at low temperature, then the melting of the double-stranded target at a higher temperature. At pH 7, while oligonucleotides **2a**, **4a** and **5a** formed triple helices with slightly lower stability than did the unmodified 22-mer **6** used as reference, the oligonucleotide **2b**, **3b**, **4b** formed triple helices with slightly higher stability (Table 2). Oligonucleotide **3a** formed triple helix with stability equivalent to that obtained with the parent phosphodiester sequence **6**, while the modifications incorporated into the oligonucleotides **5a** and **5b** have a slight destabilizing effect on the triplex stability. These results allowed us to select the modified compounds which induce the strongest stabilization in each series in order to proceed to multiple incorporations. In both series, the modified oligonucleotides with the Rp configuration at the phosphorus atom were the best candidates. In the case of linkers ending with hydroxyl groups, compound **3b** (with $n = 3$) was retained while in the case of linkers ending with amino functions modified oligonucleotide **4b** with the shortest linker ($n = 2$) was more efficient in stabilizing the triplex structure. We then studied oligonucleotides **7**, **8**, **9**, **10**, **11** and **12**, involving multiple internucleotidic linkages, at pH 7 and 5.5 in the presence of 5 mM $MgCl_2$ and 140 mM KCl in 10 mM sodium cacodylate buffer. Two types of behavior were observed depending on the pH (Table 2). At pH 7, in any case the modified oligonucleotides formed triple helices with higher stability than did the unmodified oligonucleotide **13**, used as reference (Fig. 5 shows the melting profile for the triplex containing oligonucleotide **12** at pH

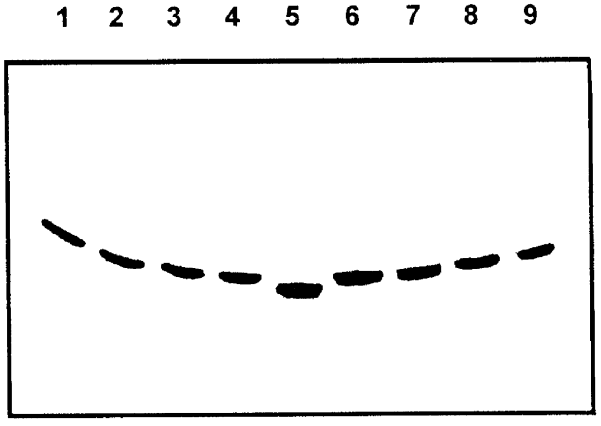


Figure 3. Denaturing polyacrylamide (20%) gel electrophoresis of modified and unmodified 22-mer oligo-2′-deoxyribonucleotides stained with methylene blue: lane 1: TFO **2a**, lane 2: TFO **2b**, lane 3: TFO **3a**, lane 4: TFO **3b**, lane 5: TFO **6**, lane 6: TFO **4a**, lane 7: TFO **4b**, lane 8: TFO **5a**, lane 9: TFO **5b**.

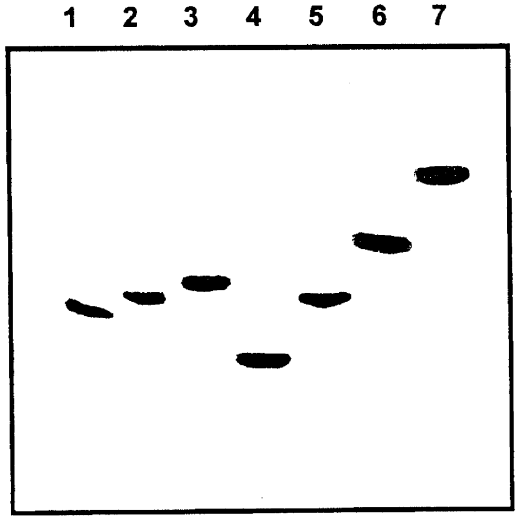


Figure 4. Denaturing polyacrylamide (20%) gel electrophoresis of modified and unmodified 17-mers oligo-2′-deoxyribonucleotides stained with methylene blue: lane 1: TFO **7**, lane 2: TFO **8**, lane 3: TFO **9**, lane 4: TFO **13**, lane 5: TFO **10**, lane 6: TFO **11**, lane 7: TFO **12**.

Table 1. Mass analyses of modified oligonucleotides **2a**–**5b** and **7**–**12**

Oligonucleotides 22-mers	Mass calculated	Mass observed	Oligonucleotides 17-mers	Mass calculated	Mass observed
2a	6670.4	6669.2	**7**	5337.75	5337.29
2b	—	—	**8**	5481.96	5481.37
3a	6684.4	6683.8	**9**	5626.18	5625.36
3b	—	—	**10**	5305.75	5306.95
4a	6668.5	6668.2	**11**	5433.97	5434.92
4b	—	—	**12**	5562.21	5562.72
5a	6682.5	6682.6			
5b	—	—			

Table 2. Melting temperatures for triplexes

Third strand	T_m^a (°C) pH = 7	Third strand	T_m^a (°C) pH = 7	T_m^b f(°C) pH = 5.5
2a	33.7	**7**	27.5	51.5
2b	38.1	**8**	28.3	54.2
3a	36.9	**9**	29.8	54
3b	38.1	**10**	26	53
4a	35.4	**11**	29	56
4b	38.6	**12**	30	58
5a	35.4	**13** (ref)	25	52.7
5b	36.1			
6 (ref)	37			

Triplex stability: T_m values of the triplexes formed by the various TFOs (**2a**–**6**) and (**7**–**13**) and the 24-bp long double-stranded target **1** (see Fig. 2 for sequences). The T_m measurements were conducted in 10 mM sodium cacodylate, 140 mM KCl and 5 mM $MgCl_2$ as described in Materials and methods. The pH of the buffer was either 7 (T_m^a) or 5.5 (T_m^b). The oligonucleotide concentrations were either 1 μmol (T_m^a) or 1.2 μmol (T_m^b). T_ms are given in °C ± 0.5 °C.

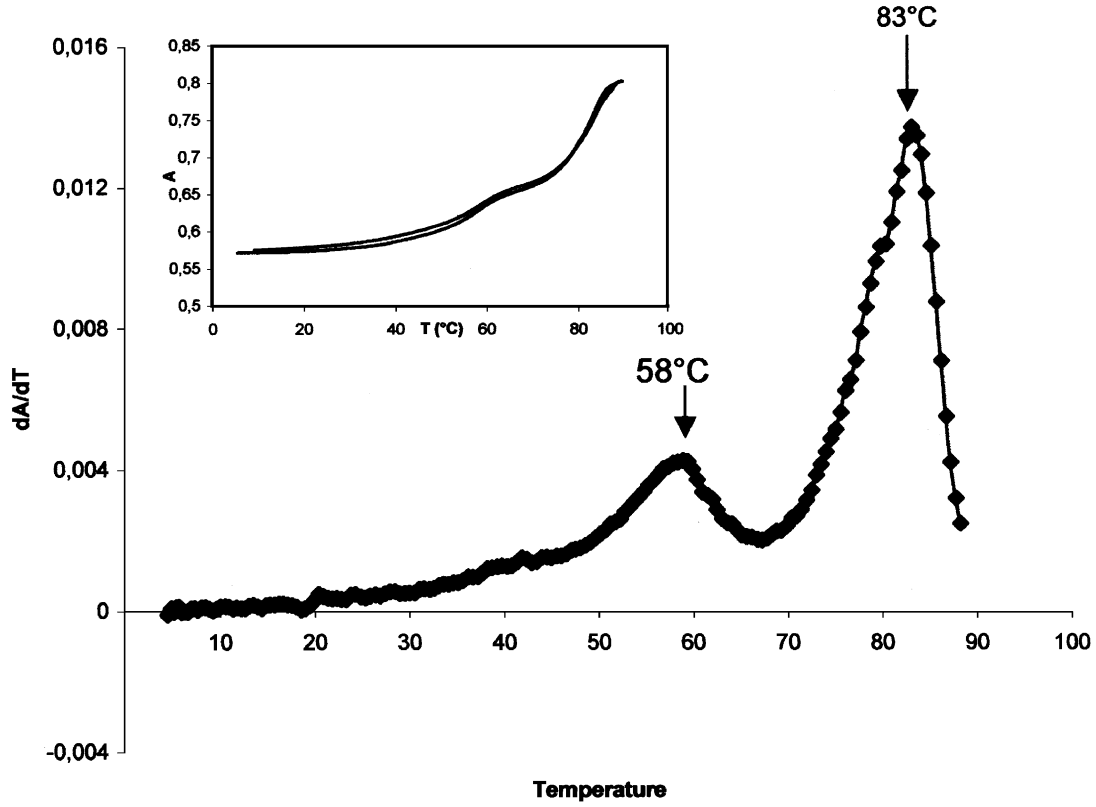


Figure 5. UV-melting profile for the triplex containing oligonucleotide **12** at pH 5.5. The ordinate shows the first derivative of the melting curve dA/dT, while the insert shows the melting curve.

5.5). We observe that in both series, either with oligonucleotides **7**, **8**, **9** (with pendant groups ending with hydroxyl functions) or with oligonucleotides **10**, **11**, **12** (with pendant groups ending with amino groups) the stability of the triplexes increased with the number of modifications whereas the T_m increase observed was not additive (probably due to the position of the modifications inside the sequence). The results are in accordance with the fact that the net global negative charge number of modified oligonucleotides decreased with the number of modified phosphates thus reducing the electrostatic repulsion between the third oligonucleotide strand and the double-stranded target. At pH 5.5, the results obtained were different. (We have verified the stability of the P–N linkage at pH 5.5 by incubating the oligonucleotide **12** in the buffer used for the hybridization studies. After 66 h incubation, the reversed-phase analysis indicated no degradation of the oligonucleotide.) In any case, the T_m values were largely higher than those observed at pH 7. However, the stabilization observed when using reference **13** confirmed that this is in part due to the protonation of the four isolated cytosines present in the sequence which remains the major contribution to the triplex stabilization. As observed at pH 7, the presence of the modified internucleotide linkages increased the stability of the triplexes except in the case of the oligonucleotide **2a**. In addition more stable triplexes were obtained with oligonucleotides involving amino functions on the pendant groups. These results are consistent with the possibility of a more extended protonation of these functions. The possibility of protonation at pH 7 of the terminal primary functions of the pendant groups in the ODNs **10**, **11** and **12** is corroborated by the ion-exchange analysis data (see Experimental). However, when comparing the T_m values in both series it is easy to see that the increase in stability due to the presence of the aminated pendant groups is weak. Examples of incorporation of large pendant groups at phosphorus have been previously reported.[21–27] Among them *N*,*N*-(dimethylaminopropyl)-phosphoramidate groups provided good triplex stabilization.[24] Different hypotheses can be raised to explain our results: (a) a problem of steric hindrance; (b) hydration changes in the major groove; (c) a loss of flexibility of the third strand and difficulty for the latter to accommodate in the major groove of the duplex; and (d) although circular dichroïsm studies have confirmed that the presence of the pendant groups in modified ODNs induced very little structural changes (see below), it is possible that the amino functions are interacting with the phosphates groups inducing slight deformations that are not favourable to the triplex stabilization.

Circular dichroism studies

Circular dichroism spectroscopy was used to compare the conformations of modified and unmodified triplexes. Chemical modifications do not introduce any new chromophores in the oligonucleotide. Therefore any cd change is expected to reflect a conformational change. Figure 6 shows cd spectra of the triplex containing the unmodified third strand **13** together with those of the triplexes containing oligonucleotides **9** and **12** at pH 5.5

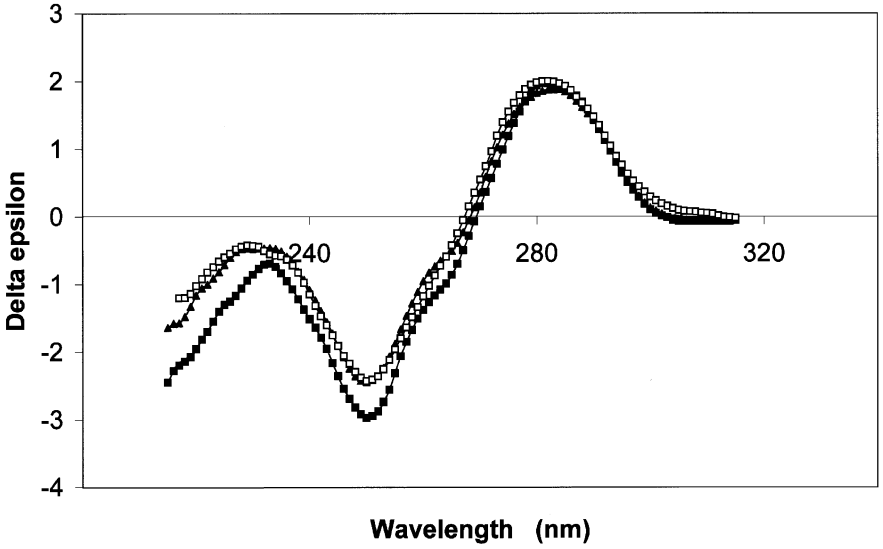


Figure 6. Cd spectra of the triplexes containing the modified oligonucleotide **9** (▲), **12** (■), and unmodified oligonucleotide **13** (□) at pH 5.5 and 20 °C.

and 20 °C. It clearly shows that the conformations of the three triplexes are quite similar and that the presence of the substituent groups on several phosphates does not modify the helix geometry. Similar results were obtained with oligonucleotides bearing two or three modifications.

Conclusion

We have described a single incorporation into a 22-mer pyrimidine TFO of a modified dinucleoside-phosphoramidate substituted with (*N*,*N*-bis-aminoethyl)-1,3-aminopropane-diamine, (*N*,*N*-bis-aminoethyl)-1,2-aminoethane-diamine, (bis-hydroxyethyl)-1,3-aminopropane-diamine, and (bis-hydroxyethyl)-1,2-aminoethane-diamine groups. Stereochimically pure Rp and Sp dinucleoside isomers were obtained and incorporated into 22-mer pyrimidine TFO. T_m measurements indicated that modified oligonucleotides involving (*N*,*N*-bis-aminoethyl)-1,2-aminoethanediamine and (bis-hydroxyethyl)-1,3-aminopropanediamine with the Rp configuration at the phosphorus atom are the best suited compounds to increase the triplex stability. Multiple (2, 3 and 4) incorporations of modified dinucleoside-phosphoramidate involving (*N*,*N*-bis-aminoethyl)-1,2-aminoethane-diamine or (bis-hydroxyethyl)-1,3-aminopropane-diamine substituent groups with the Rp configuration were performed into a 17-mer pyrimidine TFO. The binding results obtained with these 17-mer pyrimidine oligonucleotides involving a few stereochimically pure phosphate groups substituted with an (*N*,*N*-bis-aminoethyl)-1,2-aminoethane-diamine group and a double-stranded DNA target in the presence of 140 mM KCl, at pH 7 indicated that a slight increase in stability occurred and that the stabilization increased with the number of modified phosphate groups. However, this stabilizing effect is weak. Nevertheless, these results confirm literature reports[20,24,47,48] in that triplex formation is a P-stereodependant process when the third strand involves P-chiral internucleotidic modifications.

Experimental

General methods

All solvents used were dried, distilled and stored as described in ref 49. All chemicals were used as obtained unless otherwise stated. 5′-*O*-(4,4′-Dimethoxytrityl)-β-thymidine-3′-H-phosphonate was purchased from Distribio. Benzoyl chloride, diethanolamine, *N*-(2-bromoethyl)phthalimide, *N*-(3-bromopropyl)-phthalimide, *tert*-butyldimethylsilyl chloride, hydrazine hydrate, diethylenetriamine, ethyltrifluoroacetate, 2-bromoethanol, 3-bromopropanol, acetic acid, acetic anhydride, triphenylphosphine, CBr_4, pivaloyl chloride and LiN_3 were purchased from Aldrich. Hydrogen was from Air liquide. Triethylamine and sodium sulfate were purchased from Merck, 2-chloro-5,6-benzo-1,3,2-dioxaphosphorin-4-one from Fluka, pyridine and dichloromethane from SDS, and acetonitrile from Labo-Standa. Analytical thin-layer chromatography (TLC) was performed on precoated alumina plates (Merck silica gel 60F 254 (ref 5554). For flash chromatography, Merck silica gel 60 (70–230 mesh) (ref 7734) or Lichroprep Si 60, 15–25 μM (ref 109336) were used. All 4,4′-dimethoxytrityl-containing substances were identified as orange-colored spots on TLC plates by spraying with a 10% perchloric acid solution. Amino-containing substances were identified as purple-colored spots on TLC plates by spraying with a 0.1% ninhydrin solution in n-butanol. Oligonucleotide syntheses were performed on an Expedite Nucleic Acid Synthesis system 8909 from Perseptive Biosystems. Analyses and purifications by ion-exchange chromatography were carried out on a Pharmacia FPLC with a DEAE column (8 μM, 100 mm×10 mm, Waters) with a linear gradient of NaCl in 25 mM Tris/HCl buffer, pH 7 (or pH 5.5), containing 10% CH_3CN. Reversed-phase chromatography analysis was performed on a 600 E (System Controller) equipped with a photodiode array detector (Waters 990) using a Lichrospher 100 RP (5 μM) column (125 mm×4 mm) from Merck with a linear gradient of CH_3CN in 0.1 M aqueous triethylammo-

nium acetate, pH 7, with a flow rate of 1 mL/min. Mass analysis ion-molecular weights of the oligonucleotides was performed by Electrospray Mass Spectroscopy using a Quattro II (Micromas) instrument. ^{1}H NMR spectra were recorded on a Bruker AM 300WB or on a Varian Unity 500 Spectrometer and ^{31}P NMR spectra on a Varian Unity 500 Spectrometer. Absorption spectra were recorded with a Uvikon 860 spectrophotometer. The concentrations were determined using the following molar absorption coefficients $\varepsilon_{260} = 173{,}400\ M^{-1}\ cm^{-1}$ for modified and unmodified 22-mer,[50] $\varepsilon_{260} = 134{,}700\ M^{-1}\ cm^{-1}$ for modified and unmodified 17-mer,[50] $\varepsilon_{260} = 507{,}600\ M^{-1}\ cm^{-1}$ for circular double-stranded DNA target. Nucleases were purchased from Roche Biochemicals.

Preparation of the circular double-stranded target 1. Synthesis was performed according to published procedures using hexaethylene glycol linkers to tether both strands.[39,40,51]

Preparation of the bis-hydroxylated linkers 19 and 20. *N*-(2-Bromoethyl)phthalimide **14** or *N*-(3-bromopropyl)-phthalimide **15** (39, 4 mmol) and diethanolamine **16** (0.35 mol, 9 equiv) were heated at 50–60 °C for 2.5 h. Silica gel TLC analysis using a dichloromethane/methanol mixture as eluent (85:15, v/v) showed complete disappearence of the starting material ($R_{f_{14}} = 0.9$, $R_{f_{15}} = 0.9$) with formation of new spots corresponding to compounds **17** and **18** ($R_{f_{17}} = 0.44$,and $R_{f_{18}} = 0.46$). The reaction mixture was purified on a silica gel column using a dichloromethane/methanol mixture as eluent (98:2, v/v to 92:8, v/v). Yield 50%. Compounds **17** and **18** were reacted with hydrazine hydrate (2.2 equiv) in refluxing methanol for 2.5 h. During the process a white precipitate appeared. The reaction mixture was concentrated to dryness and the residue was purified on a silica gel column using methanol/concentrated ammonia mixture as eluent (97:3, v/v to 85:15, v/v). Compounds **19** and **20** were obtained as a pale yellow-colored oil with 80% yield. Selected data. Compound **19**. ^{1}H NMR ($CDCl_3$), δ ppm: 3.71 (s, 3H, OH + NH_2), 3.54 (t, $J = 9.78$ Hz, 4H), 2.75 (t, $J = 9.78$ Hz), 2.54 (m, 6H). Mass analysis. I.S. polarity positive. Compound **19**. Calculated for $C_6\ H_{16}\ N_2O_2$: M + H = 149, 21. Found 148.9.

Preparation of bis-protected aminated linkers 30 and 31. Diethylene triamine **21** (50 mmol) and ethyltrifluoroacetate **22** (170 mmol, 3.5 equiv) were refluxed in acetonitrile (100 mL) containing water (1.1 mL, 1.2 equiv) for 18 h. The reaction mixture was concentrated to dryness and the residue was then taken in CH_2Cl_2 (100 mL). Compound **23** appeared as a white precipitate and was recovered by filtration and dried over P_2O_5. Yield 95%. $R_{f_{23}} = 0.70$ using a dichloromethane/methanol mixture as eluent (80:20, v/v). ^{1}H NMR ($CDCl_3$), δ ppm: 2, 58 [m, 4H, $(CH_2)_2$N], 3, 38 (m, 4H, 2× CH_2NHCO).

Compound **23** (9.8 mmol) was placed in CH_3CN (15 mL) and excess triethylamine was added (14.7 mmol, 1.5 equiv). The mixture was heated to 50 °C and bromoethanol **24** (9.8 mmol, 1 equiv) or bromopropanol **25** was added. The heating was continued for 24 h, then the solvent was removed by evaporation and the residue was dissolved with $CHCl_3$. A small amount of insoluble material was removed by filtration and the filtrate concentrated to dryness. The residue was purified by silica gel chromatography using a dichloromethane/methanol mixture as eluent (97:3, v/v to 94:6, v/v). Compounds **26** and **27** were obtained with 50% yield. $R_{f_{26}} = 0.38$, $R_{f_{27}} = 0.35$ using a dichloromethane/methanol mixture as eluent (90:10, v/v).

Selected data: compound **26**. ^{1}H NMR ($CDCl_3$), δ ppm: 2.7 (m, 6H, –$(CH_2)_3$ N), 3.0 (br s, 1H, OH), 3.4 (q, $J = 5.5$ Hz, 4H, CH_2NHCO), 3.65 (t, $J = 5.2$ Hz, 2H, CH_2OH), 7.82 (br s, 2H, CONH). Mass analysis. I.S. polarity positive. Compound **26** calculated for $C_{10}\ H_{15}\ F_6\ N_3O_3$. M + H = 339. Found 339.1. Compound **27** Calculated for $C_{11}\ H_{17}\ F_6\ N_3O_3$. M + H = 354. Found 354.7.

Compounds **26** and **27** (5.9 mmol each) were dried in a dessicator overnight as well as triphenylphosphine and lithium azide, separately. The next day, compound **26** or **27** (5.9 mmol), triphenylphosphine (8.8 mmol) and lithium azide (17.7 mmol, 3 equiv) were placed with anhydrous DMF in a round-bottomed flask. Carbon tetrabromide (8 mmol, 1.5 equiv) solubilized in DMF (5 mL) were added dropwise to the flask. The reaction was exothermic. After 2 h reaction at room temperature, MeOH (2 mL) was added and the reaction mixture was evaporated to dryness. The residue was purified on a silica gel column using a dichloromethane/ethylacetate mixture as eluent (90:10, v/v to 80:20, v/v). Compounds **28** and **29** were obtained as oil. Yield 70%. $R_{f_{28}} = 0.60$, and $R_{f_{29}} = 0.57$ using a dichloromethane/acetone mixture as eluent (80:20, v/v). Selected data. Compound **28**. ^{1}H NMR ($CDCl_3$), δ ppm: 2.7 (m, 6H, $(CH_2)_3$ N), 3.4 (m, 6H, 2 CH_2NHCO, CH_2N_3), 7.2 (br s, 2H, CONH). Mass analysis. I.S. polarity positive. Compound **28**: Calculated for $C_{10}H_{14}F_6N_6O_2$. M + H = 365.25. Found 365.1. Compound **29:** Calculated for $C_{11}H_{16}F_6N_6O_2$. M + H = 379.25. Found 379.

Compounds **28** and **29** (4.12 mmol) were separately dried by co-evaporation with anhydrous acetonitrile (4×15 mL) and then solubilized in MeOH (30 mL). Then under argon, 10% palladium on activated charcoal (0. 15 g) was added and hydrogenation was performed for 18–24 h under magnetic stirring. The catalyst was removed by filtration and the filtrate was concentrated to dryness to give compounds **30** and **31** as pale yellow-colored oils. Yields 70%. Positive test with ninhydrin. Products were used in the next steps without purification. Mass analysis. I.S. polarity positive. Compound **30**. Calculated for $C_{10}H_{16}F_6N_4O_2$. M + H = 339.25. Found 339.1. Compound **31**. Calculated for $C_{11}H_{18}F_6N_4O_2$. M + H = 353. Found 353.1.

Preparation of the modified dinucleosides 32a and 32b. The synthesis was achieved as reported in ref 42. The purification and separation of the dinucleosides **32a** and **32b** were performed in a single step on a silica gel col-

umn (60H Merck) using ethylacetate/acetic acid mixture as eluent (99.9:0.1, v/v). The isomer **32a** was eluted first. Thin-layer chromatography analysis confirmed the full separation of the isomers. $R_{f_{32a}} = 0.5$, $R_{f_{32b}} = 0{,}34$ using a dichloromethane/methanol mixture as eluent (90:10, v/v). The purity of each isomer was confirmed by ^{31}P NMR analysis in $CDCl_3$ (external reference H_3PO_4). One single peak was obtained for each isomer $\delta_{32a} = 7.25$ ppm and $\delta_{32b} = 8.54$ ppm.

Preparation of modified dinucleosides involving protected bis-hydroxylated linkers 33a–38b. Each pure isomer **32a** and **32b** of the dinucleoside-H-phosphonate 5′-*O*-(4,4′-dimethoxytrityl)-thymidylyl-3′-*O*-*tert*-butyl-dimethylsilyl-thymidine-3′-H-phosphonate (1*g* each, 1.05 mmol), previously dried by co-evaporation with anhydrous pyridine (5 mL, three times), was dissolved in a CH_3CN/pyridine mixture (50:50 v/v) (8 mL) and treated separately with bis-hydroxylated linkers **19** ($n = 2$) or **20** ($n = 3$) (7.0 mmol) solubilized by an anhydrous pyridine/CCl_4 mixture (50:50, v/v) (5 mL) for 10 min over argon atmosphere. Silica gel TLC analysis using a dichloromethane/methanol mixture as eluent (90:10, v/v) showed complete reaction with formation of a new spot. The reaction mixture was concentrated to dryness and solubilized with CH_2Cl_2 (30 mL). The organic phase was washed with $NaHCO_3$ (5 mL) and dried over Na_2SO_4. Silica gel TLC analysis using a dichloromethane/acetone-/triethylamine mixture as eluent (50:50:10, v/v/v) (two elutions) showed complete reaction with the formation of a new spot ($R_{f_{33a}} = 0.46$, $R_{f_{33b}} = 0.55$, $R_{f_{34a}} = 0.36$, and $R_{f_{34b}} = 0.43$). The organic phase was concentrated to dryness and the residue was purified on a silica gel column using a dichloromethane/methanol mixture (97:3, v/v to 85:15, v/v) containing 0.1% triethylamine as eluent. Yields 55%.

Dinucleosides **33a**, **33b**, **34a**, and **34b** (0.55 mmol) were co-evaporated with anhydrous pyridine (20 mL, three times) and during the last evaporation, 10 mL of pyridine were kept in the flask. The solution was cooled to 0 °C and benzoyl chloride (1.25 mmol) was added dropwise under stirring and argon atmosphere. After 30 min, the reaction was allowed to react at room temperature for 3 h. The reaction was monitored by TLC analysis using a CH_2Cl_2/MeOH mixture as eluent (90:10, v/v) showing the formation of new products with higher R_f values ($R_{f_{35a}} = 0.44$, $R_{f_{35b}} = 0.46$, $R_{f_{36a}} = 0.53$, and $R_{f_{36b}} = 0.56$).[($R_{f_{33a}} = 0.17$, $R_{f_{33b}} = 0.20$, $R_{f_{34a}} = 0.13$, and $R_{f_{34b}} = 0.15$]. When the reaction was completed, the reaction mixture was hydrolyzed with $NaHCO_3$ (4 mL), extracted with dichloromethane. The organic phase was dried over Na_2SO_4, evaporated to dryness and the residue was purified on a silica gel column using a dichloromethane/methanol mixture (98:2, v/v to 93:7, v/v) containing 0.1% triethylamine as eluent. Yield 75%.

The fully protected dinucleosides **35a**, **35b**, **36a**, and **36b** (0.40 mmol) were treated under stirring with a 1.1 M tetrabutylammonium fluoride solution in tetrahydrofuranne (5 equiv) for 1.5 h. The reaction was monitored by TLC analysis using a CH_2Cl_2/MeOH mixture as eluent (90:10, v/v) showing the formation of new products with lower R_f values ($R_{f_{37a}} = 0.40$, $R_{f_{37b}} = 0.44$, $R_{f_{38a}} = 0.39$, and $R_{f38b} = 0.42$). When the reaction was completed, the reaction mixture was hydrolyzed with water (0.4 mL), extracted with dichloromethane. The organic phase was washed with water, dried over Na_2SO_4, evaporated to dryness and the residue was purified on a silica gel column using a dichloromethane/methanol mixture (97:3, v/v to 95:5, v/v) containing 0.1% triethylamine as eluent. Yield 80%. Mass analysis. I.S. polarity positive. Compounds **37a**. and **37b**. Calculated for $C_{61}H_{67}N_6O_{17}P$. $M + H = 1187$. Found: **37a** 1187.4 and **37b** 1187.4. Compounds **38a**. and **38b**. Calculated for $C_{62}H_{69}N_6O_{17}P$. $M + H = 1201$. Found: **38a** 1201.6 and **38b** 1201.4.

Preparation of phosphoramidite derivatives 39a–40b of the modified dinucleosides 37a–38. Compounds **37a**–**38b** (0.32 mmol) were dried by co-evaporation with anhydrous pyridine (5 mL) then with anhydrous CH_3CN (5 mL, three times) and left in a dessicator under vacuum overnight. The next day, the dessicator was filled with argon before its opening. The residue was solubilized with 1,2-dichloroethane (8 mL) and diisopropylethylamine (0.2 mL, 0.09 g, 3 equiv, 0.7 mmol) was added and then 2-cyanoethyl diisopropylchlorophosphoramidite (0. 094 mL, 0.1 g, 1.3 equiv, 0.42 mmol) was added dropwise under stirring at room temperature. After 11.5 h, silica gel TLC analysis using a ethylacetate/acetone/triethylamine mixture as eluent (75:25:5, v/v/v) showed nearly complete reaction with the formation of two new spots corresponding to both isomers of the phosphoramidite derivative. The reaction mixture was diluted with cold ethyl acetate (30 mL) previously washed with a cold aqueous 10% sodium carbonate solution. The organic phase was washed with a cold 10% aqueous sodium carbonate solution (10 mL) and with a cold saturated aqueous sodium chloride solution (10 mL), dried over Na_2SO_4 and concentrated to dryness. The residue was purified on a silica gel column using ethylacetate/acetone/triethylamine mixture as eluent (75:25:5, v/v/v). After precipitation from cold (−78 °C) hexane, compounds **39a**–**40b** were obtained as a white powder. Yields 70–75%.

Preparation of the modified dinucleosides involving the protected bis-aminated linkers 41a–44b. The fully protected dinucleotides **41a**–**42b** were obtained as reported for the preparation of dinucleotides **33a**–**34b** by replacing the bis-hydroxylated linkers **19** ($n = 2$) or **20** ($n = 3$) with the bis-aminated linkers **30** ($n = 2$) or **31** ($n = 3$). Silica gel TLC analysis using a dichloromethane/methanol mixture as eluent (90:10, v/v) showed complete reaction with a new spot ($R_{f_{41a}} = 0.40$, $R_{f_{41b}} = 0.44$, $R_{f_{42a}} = 0.41$, $R_{f_{42b}} = 0.45$). Yield = 70% after purification on a silica gel column using a dichloromethane/methanol mixture as eluent (98:2, v/v to 95:5, v/v). ^1H NMR ($CDCl_3$), δ ppm: 7.76–6.78 (m, 21H Ar), 5.11 (m, 1H, H-1′), 4.93 (br s, 1H, NH), 4.36 (d, 2H, $J = 6.77$ Hz, O-CH_2), 4.0–3.98 (m, 1H, OH), 3.77–3.66 (m, 6H, H-3′, H-4′, H-5′, H-5″ + CH_2O), 3.75 (s, 6H, OCH_3), 3.12–3.07 (m, 2H, CH_2N), 2.17–2.02 (m, 2H, H-2′, H-2″), 1.61–1.56 (m, 2H, CH_2).

Starting from dinucleotides **41a**–**42b** the desilylated dinucleotides **43a**–**44b** were obtained as reported for the preparation of dinucleotides **37a**–**38b**. Purification was performed on a silica gel column using a dichloromethane/methanol (98:2, v/v to 97:3, v/v) mixture containing 0.3% triethylamine as eluent. Yield 70–75%. [$R_{f_{43a}} = 0.30$, $R_{f_{43b}} = 0.32$, $R_{f_{44a}} = 0.27$, $R_{f_{44b}} = 0.30$ using a dichloromethane/methanol mixture as eluent (90:10, v/v)].

Mass analysis. I.S. polarity positive. Compounds **43a** and **43b**. Calculated for $C_{51}H_{59}F_6N_8O_{15}P$. M + H = 1169. Found: **43a**: 1169.5 and **43b**: 1169.3. Compounds **44a**. and **44b**. Calculated for $C_{52}H_{61}F_6N_8O_{15}P$. M + H = 1183. Found: **44a**: 1183.3 and **44b**: 1183.5.

Preparation of phosphoramidite derivatives 45a–46b of the modified dinucleosides 43a–44b. Starting from dinucleosides **43a**–**44b** and proceeding as reported for the preparation of phosphoramidite derivatives **39a**–**40b**, except for the purification on silica gel column which was omitted in order to prevent partial loss of the trifluoroacetyl groups, the phosphoramidite derivatives **45a**–**46b** were obtained with 75% yield. Foam. CCM: $R_f = 0.65$ using a mixture of ethyl acetate, acetone and triethylamine as eluent (75:25:5, v/v/v).

Preparation of oligonucleotides 2a–5b and 7–12. Oligonucleotides were synthesized on a 1-μmol scale on an Expedite Nucleic Acid Synthesizer using standard β-cyanoethyl phosphoramidite chemistry.[52] Reagents were prepared as described in the user manual and a standard concentration of a phosphoramidite solution was used for all the umodified nucleotides. Concentrations of the modified dinucleoside-phosphoramidites involving varied substituent groups on the internucleotidic phosphate were increased up to 1.5 times that of the umodified monomers. The coupling time for the modified monomers and the detritylation time after their incorporation were increased by 50 and 20%, respectively. Coupling efficiency was estimated from trityl assays and were >95% per step for the modified dinucleosides. After the chain assembly (trityl-on mode), oligomers **4a**–**5b** and **10**–**12** attached to the support were treated with *tert*-butylamine in acetonitrile, to selectively remove the β-cyanoethyl groups from the phosphates so as to prevent the formation of acrylonitrile adducts on the primary amino functions of the pendant groups. They were then removed from the solid support and deprotected at base level by concentrated aqueous ammonia treatment (28% aq NH_3) (4 mL) at room temperature overnight. Oligonucleotides **2a**–**3b** and **7**–**9** were only treated by 28% aq NH_3 (4 mL) at room temperature overnight. The ammonia solution was then removed by evaporation. Purification of the tritylated oligonucleotides was performed by ion exchange chromatography on a DEAE column (8 μM, 100 mm×10 mm, Waters) using a linear gradient of NaCl (0.075–0.525 M over 30 min) in a 25 mM Tris/HCl buffer containing 10% CH_3CN, pH 7. The desalting step was performed on a HR 10/10 column (Pharmacia) packed with Lichroprep PR 18 (Art 13900 from Merck). After the desalting step, oligonucleotides were detritylated by aqueous acid acetic treatment (80%) for 20 min at room temperature. Acetic acid and tritanol were then removed following standart conditions. The purity of all the oligo-2′-deoxyribonucleotides described was verified by reversed-phase analysis using a Lichrocart system (125×4 mm) packed with Lichrospher RP 18 (5 μm) from Merck eluted with a linear gradient of acetonitrile from 0 to 27.5% over 30 min, in a 0.1 M aqueous triethylammonium acetate buffer, pH 7, with a flow rate of 1 mL/min. Ion exchange analysis was also performed on a Mono Q column (HR 5/5, Pharmacia) using a linear gradient of NaCl (0.0–0.6 M over 60 min) in a 25 mM Tris/HCl buffer containing 10% CH_3CN, pH 7. As expected unmodified ODN **13** was eluted with the highest retention time 38 min 18 s. and ODNs containing the pendant groups with amino functions were less retained than those containing the hydroxyls functions (R_{t_7} = 29 min 36 s; $R_{t_{10}}$ = 26 min 18 s; R_{t_8} = 25 min 24 s; $R_{t_{11}}$ = 19 min 6 s; R_{t_9} = 18 min 18 s; $R_{t_{12}}$ = 9 min 30 s). These results are in accordance with the possible protonation of the primary amino functions at the ends of pendant groups in oligonucleotides **10**, **11** and **12**. Analysis by 20% polyacrylamide gel electrophoresis using denaturing conditions (7 M urea) indicated (Figs 3 and 4) the presence of only one band for each modified oligonucleotide. In the both series, either with oligonucleotides **7**, **8**, **9** (with pendant groups ending with hydroxyl functions) or with the oligonucleotides **10**, **11**, **12** (with pendant groups ending with amino groups) mobilities were different according to the number of modifications (Fig. 4). Modified oligonucleotides with the greatest number of modifications were more retained in the gel and taking into consideration the same number of modifications, oligonucleotides involving the pendant groups with terminal amino functions were more retained than the corresponding hydroxylated oligonucleotides. The overall yield after the synthesis and purification steps is close to 40% for oligonucleotides with a single phosphate modification and 30–35% for oligonucleotides involving multiple phosphate modifications.

Full deprotection and nucleoside composition of the modified oligonucleotides **2a**–**5b** were ascertained by nuclease degradation. An aliquot of the oligonucleotide was digested with snake venom phosphodiesterase (Pharmacia Biotech) and alkaline phosphatase (Boehringer) in a 0.1 M Tris–HCl buffer, pH 8.2, at 37 °C. After inactivation of the enzyme at 90 °C for 2 min, the digestion products were analyzed by reversed-phase chromatography using a Lichrocart system (125×4 mm) packed with Nucleosil 100-5 C18 from Macheray-Nagel equilibrated with a 0.1 M aqueous triethylammonium acetate buffer, pH 7. The column was eluted at a flow rate of 1 mL/min with 0.1 M aqueous triethylammonium acetate buffer, pH 7, for 10 min and then with a linear gradient of 0–24% of acetonitrile in a 0.1 M aqueous triethylammonium acetate buffer, pH 7, for 30 min and with a linear gradient of 24–56% of acetonitrile in a 0.1 M aqueous triethylammonium acetate buffer, pH 7, for 20 min. Detection was performed at 260 nm. Oligonucleotides were totally degraded. Three peaks were obtained. Comparison with natural nucleosides and modified dinucleosides (obtained after full deprotection of sample dinucleosides **37a**–**38b** and **43a**–**44b**) allowed

us to identify the different peaks. Mass analysis results confirmed the calculated mass values for each oligonucleotide (Table 1).

T_m measurements by UV absorption experiments

Changes in absorbance with temperature of 1 μM triplexes in a 10 mM sodium cacodylate buffer (pH 5.5, or pH 7) containing 5 mM $MgCl_2$ and 140 mM KCl were measured at $\lambda = 260$ nm in a Uvikon 941 cell changer spectrophotometer equipped with a Huber PD 415 temperature programer connected to a cryothermostat ministat circulating water bath (Huber). A cacodylate buffer was chosen due to its limited dependence of pH on temperature. The temperature was regulated at a rate of 0.2 °C/min from 0 to 80 °C. To monitor triplex-to-duplex transition, the samples were first heated and then cooled. Oligonucleotide concentrations were 1 μM of the circular duplex, and either 1 or 1.2 μM of the third strand (see sequences in Fig. 2). The absorbance at 260 and 340 nm was recorded every 5 min. Corrections for spectrophotometric instability were made by substracting the absorbance at 340 nm from that at 260 nm. The T_m values were determined using the first derivative. Estimated accuracy of the melting temperature is ±0.5 °C.

Circular dichroism experiments

Cd measurements were carried out on a Jobin Yvon Mark IV dichrograph in optical cells with a pathlength of 0.5 or 1 cm. Each cd spectrum was an average of two scans with the buffer blank subtraction. The concentration used to calculate the cd intensity was that of the nucleotide unit.

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