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A New Solid-phase Synthesis of Oligonucleotides 3′-conjugated with Peptides

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Abstract—A convenient 'on line' solid-phase synthesis of oligonucleotides conjugated at the 3′-end with peptides by means of a polymeric support linking the first nucleoside via the base has been developed. A 17-mer designed for antisense experiments against HIV-1, linking at the 3′-terminus the tripeptide Gly-Gly-His, was prepared in good yields and characterized by MALDI-TOF mass spectrometry.

Introduction

Therapeutic applications of synthetic, not modified oligodeoxyribonucleotides (ODNs) in antisense strategy are usually dramatically limited by their modest cellular uptake and the rapid enzymatic degradation.[1,2] Covalent attachment of peptide residues to synthetic oligonucleotides is of great value not only to enhance their resistance to nucleases, but also for the peculiar functions imparted by the peptide. For instance, a sensible increase in cell permeability can be easily achieved by conjugating to ODNs hydrophobic aminoacids or cationic residues, such as polylysine, or nuclear transport signals peptides.[3–5] Another interesting application of peptides is related to their ability to act as artificial nucleases. For instance, small peptide sequences as Gly-Gly-His or His-Gly-His have been shown[6] to mimic the active site of RNase A catalyzing, after Cu(II) or Ni(II) complexation, the hydrolytic cleavage of single stranded nucleic acids. By covalently linking these peptides to antisense ODNs, the nucleolytic activity can be therefore directed towards any desired, specific sequence of the target RNA.[7] In principle, any further reactive functional group, either of the bases or of the sugar-phosphate backbone of the oligonucleotide, can be profitably exploited for the conjugation with peptides; however a chain terminal derivatization is generally preferred, basically for two reasons: (1) this kind of conjugation does not impair the hybridization properties of the ODN; and (2) it is not necessary to prepare modified nucleosides as building blocks to be used in the ODN standard chain assembly.

For in vivo experiments, conjugation at the 3′-end is generally required, since this induces resistance to 3′-exonucleases, the most abundant nucleases in cells. From a strictly synthetic point of view, linking a label, such as a peptide fragment, at the 3′-end of an oligonucleotide following an 'on line' strategy[7–10] is more troublesome than attaching it at the 5′-OH terminus; in fact, for 3′-conjugations, ad hoc protocols have to be adopted, based on the usage of not commercially available solid supports and suitable spacers, connecting the two fragments, which have to be proven compatible with the chemistry used in the synthesis of peptides and of oligonucleotides.

In the present work we report on the synthesis and the usage of a modified solid support (**6**, Scheme 1) which, linking the first nucleoside unit via the nucleobase, allows the standard automated phosphoramidite elongation of the oligonucleotide chain from the 5′-OH function (Scheme 2) and, successively, at the 3′-end, the growing of the desired peptide sequence by Fmoc strategy. Small hybrids have been prepared and fully characterized to optimize the synthetic protocol; furthermore, a 17-mer linking at the 3′-end the tripeptide Gly-Gly-His, to be tested in in vitro experiments as an antisense and RNA-cleaving agent against HIV-1, has been synthesized in good yields and characterized by MALDI-TOF mass spectrometry.

Key words: Nucleic acids; peptides; solid-phase synthesis; HIV.
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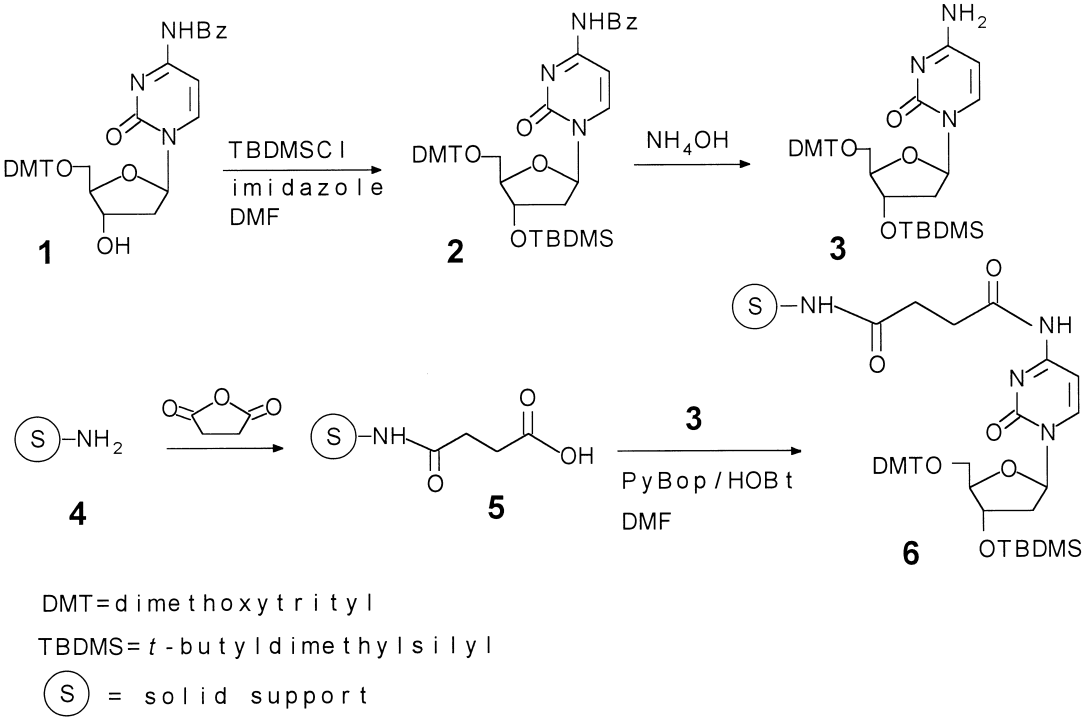
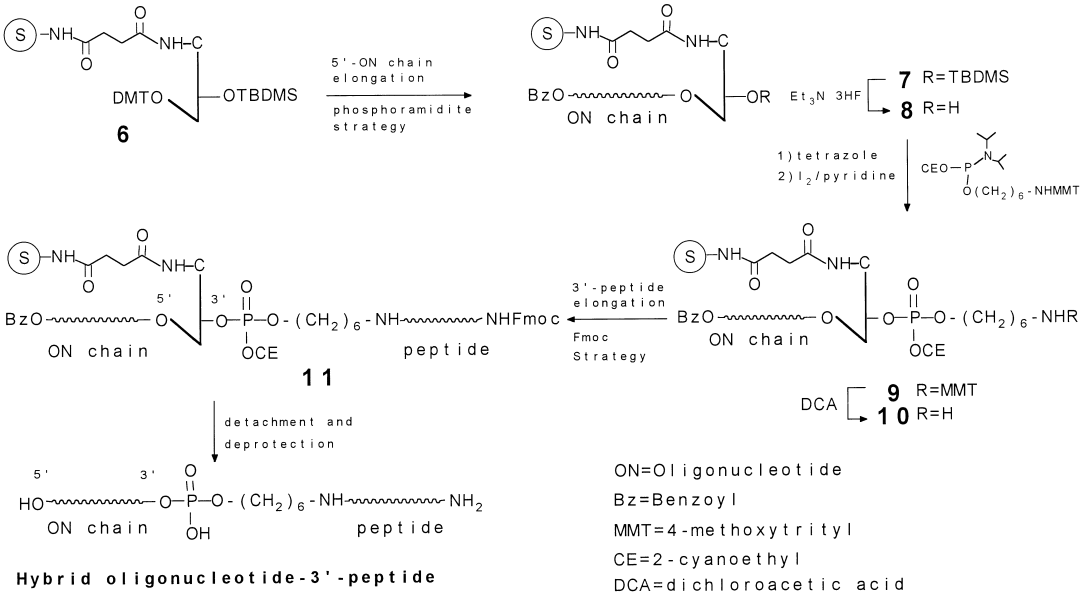
Results and Discussion

Common requirement to the already reported procedures[7–10] for the 'on line' synthesis of ODNs conjugated at the 3′-end with peptides is the preparation of ad hoc derivatized solid supports linking a suitable spacer, selectively cleavable at the end of the elongation, to attach the peptide fragment. This is then coupled with a second linker unit, stable to the successive treatments, assuring the covalent attachment of the first nucleotide of the ODN chain, synthesized following standard automated procedures. The compatibility of the chemical methods to synthesize the peptide and the ODN chains and the careful choice of the protecting groups for the aminoacids side chain moieties are two basic points for the design of ODN-peptide conjugates. Moreover the feasibility of this synthetic approach depends also on the appropriateness of the spacers and of the conditions for the final deprotection and detachment of the synthesized hybrids. These problems, including possible racemizations, have been faced by several groups who used different supports, combining either Fmoc or Boc strategies with the classical phosphoramidite chemistry for the 'on line' synthesis of



Scheme 1.



Scheme 2.

ODN-peptide hybrids. We developed a straightforward, high yield synthetic protocol to obtain ODNs conjugated at the 3′-end with peptides, exploiting as a solid support a modified Tentagel resin, a polystyrene-polyethylene glycol copolymer of widespread use in the solid-phase synthesis of peptides[11] and oligonucleotides.[12] In this new support, that we have previously reported[13] for the preparation of ODNs containing a 3′-3′ phosphodiester bond, the first nucleoside unit is anchored to the matrix via the nucleobase, so that both the 5′-OH and the 3′-OH ends, orthogonally protected, are potentially available for successive functionalizations with phosphoramidite derivatives: the 5′-hydroxyl is used for the classical ODN chain assembly in the 3′-5′ direction, while the 3′-OH function can be profitably exploited for the derivatization with a commercially available amino linker and then coupled with suitably N- and side chain protected aminoacids following standard methods.

The preparation of functionalized support **6** has been efficiently achieved starting from 5′-O-DMT-N^4-benzoyl-2′-deoxycytidine (**1**, Scheme 1), which was first converted into the corresponding 3′-TBDMS nucleoside **2** and then deprotected at the cytosine exocyclic amino function by ammonia treatment, giving **3** (92% overall yield). Tentagel^R resin **4** (0.22 mequiv/g of free amino groups), prefunctionalized with succinic anhydride to yield **5**, was reacted with cytidine derivative **3** in the presence of PyBop/HOBt in DMF/DIEA affording support **6** with a nucleoside loading of 0.14 mequiv/g.

The synthetic route to obtain the desired 3′-conjugated ODNs is outlined in Scheme 2. The synthesized solid support **6** (Scheme 2) has been exploited first for the standard automated ODN chain assembly; once completed the desired sequence, a final capping of the 5′-OH end has been carried out by treatment with benzoyl chloride in pyridine obtaining **7**. Removal of the TBDMS group from the 3′-OH function by reaction with $Et_3N{\cdot}3HF$ (18 h, rt) allowed the deprotection of the 3′-OH end, giving **8**. This was then reacted with the commercially available phosphoramidite derivative of the chosen linker 6-(4-monomethoxytrityl)-aminohexan-1-ol, giving **9**. The incorporation of the linker molecule resulted to be quantitative as determined by spectroscopic measurements of the 4-monomethoxytrityl cation released by acidic treatments of weighed amounts of support **9**. After deprotection of the 3′-amino terminus by DCA (10% in CH_2Cl_2), the synthesis of the peptide fragment was carried out on **10** following the standard Fmoc strategy.[14] Detachment from solid support **11** and full deprotection of the synthesized hybrids were achieved by addition of a 0.1 M NaOH solution (24 h, rt), conditions which were proven to be non-racemizing.[15]

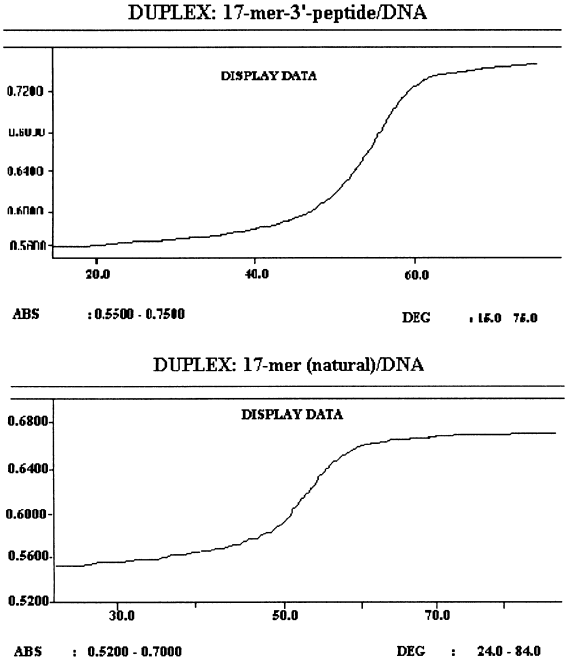


Figure 1. UV Melting profiles. Concentration approximately 2 μM each strand buffer: 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.

To test the feasibility of this pathway, two small hybrids, $^{5'}AC^{3'}$-linker-Val (**a**) and $^{5'}AC^{3'}$-linker-Val-Phe (**b**) were synthesized and purified by HPLC; their structures were confirmed by ^1H NMR and FABMS data.

Following this synthetic route, we then prepared the 17-mer $^{5'}$CTG-CTA-GAG-ATT-TTT-AC$^{3'}$, complementary to a tract of the PBS region of genomic RNA of HIV-1, conjugated at the 3′-end with the tripeptide Gly-Gly-His. After synthesis of the oligonucleotide sequence and insertion of the amino linker, leading to 3′-functionalized support **9**, the support was used for the peptide synthesis, performed by addition of *N*-Fmoc protected α-aminoacids in the presence of PyBop/HOBt as activating system. After each coupling step, an acetylation has been carried out to cap the unreacted amino groups. The histidine residue has been reacted with the support as *N*-α-Fmoc-*N*-im-trityl-histidine; the imidazole deprotection was obtained by DCA treatments, which allowed to quantitate the incorporation of the tripeptide (55% with respect to the initial nucleoside functionalization). The crude detached conjugate was finally purified by HPLC on an anion exchange Nucleogen column. Oligomer **c** was desalted by gel filtration chromatography on a Sephadex G25 column eluted with H_2O and the isolated product was checked for purity by HPLC on a RP18 column, showing the isolated compound to be more than 98% pure. MALDI-TOF mass spectrometry was employed to characterize the synthesized conjugate.

Thermal denaturation experiments have been carried out to verify whether the presence of the peptide fragment Gly-Gly-His, conjugated at the 3′-end of the 17-mer, interfered negatively in the binding of the oligonucleotide with complementary DNA and RNA fragments. As evaluated by comparison of the melting temperatures relative to the duplexes formed by the 17-mer-linker-Gly-Gly-His with the complementary DNA and RNA 17-mers and a DNA 24-mer, with those of the duplexes formed by the corresponding not conjugated 17-mer (see Table 1), we found that the affinity of the oligonucleotide towards target DNA or RNA sequences was not hampered by the 3′-peptide tail.

Table 1. Melting temperatures of the studied duplexes

Duplex	Tm(°C)
17-mer-3′-peptide (c)/DNA(17-mer)	52.6
17-mer (natural)/DNA (17-mer)	52.6
17-mer-3′-peptide (c)/RNA (17-mer)	55.9
17-mer (natural)/RNA (17-mer)	56.0
17-mer-3′-peptide (c)/DNA (24-mer)	54.4
17-mer (natural)/DNA (24 mer)	54.0

DNA (17-mer): 5′-GT-AAA-AAT-CTC-TAG-CAG-3′
RNA (17-mer): 5′-GU-AAA-AAU-CUC-UAG-CAG-3′
DNA (24-mer): 5′-$(T)_7$-GT-AAA-AAT-CTC-TAG-CAG-3′

In vitro and in vivo experiments to investigate the pharmacological potential of such compound against HIV-1, either as antisense agent or as artificial nuclease, are currently underway also in collaboration with specialized laboratories.

Conclusion

An alternative and efficient solid-phase method for the synthesis of oligonucleotides conjugating peptide fragments at the 3′-end, a class of modified oligomers not easily accessible by ordinary synthetic methods, the properties of which are not yet completely explored, has been developed. To this purpose, a modified Tentagel support (**6**), derivatized with a base-linked nucleoside having the 5′- and the 3′-hydroxyls temporarily protected by the selectively removable DMT and TBDMS groups, has been conveniently exploited. This kind of support is potentially of use also for the preparation of 5′,3′-diconjugated ODNs. We have optimized a synthetic protocol in which the 3′-linked peptide fragment was assembled after the ODN chain. As a linker molecule connecting the oligonucleotide and the peptide fragments, a commercially available phosphoramidite derivative of 6-(4-monomethoxytrityl)-aminohexan-1-ol has been quantitatively attached to the 3′-OH end of the oligonucleotide chain. The terminal amino function has been then deprotected by acidic treatment and used for the peptide assembly using Fmoc-protected α-aminoacids as building blocks. Following only slightly modified standard automated phosphoramidite and Fmoc procedures, two small hybrids have been synthesized and characterized by ^{1}H NMR and FABMS. The 17-mer $^{5'}$CTG-CTA-GAG-ATT-TTT-AC$^{3'}$, complementary to a tract of the PBS region of genomic RNA of HIV-1, conjugated at the 3′-end with the tripeptide Gly-Gly-His, to be tested in antisense experiments, has been prepared in good yields and characterized by MALDI-TOF mass spectrometry. Thermal denaturation data showed that the peptide tail at the 3′-end of the oligonucleotide did not destabilize the duplexes formed by the 17-mer with complementary DNA and RNA sequences.

Experimental

Materials and methods

NMR spectra were recorded on Bruker WM-400 and WM-270 spectrometers. All chemical shifts are expressed in ppm with respect to the residual solvent signal. Tentagel^R resin was purchased from Rapp Polymere, Tubingen, Germany. The solid support functionalizations with derivative **3** were carried out in a short glass column (5 cm length, 1 cm id) 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′-deoxyribo- or ribonucleosides as building blocks. In the case of not modified oligomers (17-mer and 24-mer sequences complementary to 17-mer **c**), the synthesis and purification were carried out following a standard protocol.[16] Peptide synthesis was performed using *N*-Fmoc protected aminoacids purchased from NovaBiochem. 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: synthesis of support 6

The synthesis and characterization of nucleoside derivative **3** have been carried out as we previously reported.[13] 1.0 g of Tentagel^R resin **4** (0.22 mmol of free amino groups per g), mixed with 1.0 g (10 mmol) of succinic anhydride in 8 mL of anhydrous pyridine, was shaken at rt for 16 h. Resulting support **5** was filtered and exhaustively washed with pyridine, $CHCl_3$ and Et_2O and dried under reduced pressure. **5**, which resulted to be negative to Kaiser test, was then reacted with 655 mg (0.96 mmol) of **3** dissolved in 4 mL of anhydrous DMF, in the presence of 270 mg (0.5 mmol) of PyBop, 70 mg (0.5 mmol) of HOBt and 170 μL of DIEA for 16 h at rt under shaking. After exhaustive washings with CH_2Cl_2, CH_3OH and Et_2O, solid support **6** was dried under reduced pressure. The functionalization of the resin with derivative **3** resulted to be 0.14 mequiv/g, as estimated by spectroscopic measurements ($\lambda = 498$ nm; $\varepsilon = 71700$ $cm^{-1}\,M^{-1}$) 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.

Synthesis of hybrid a ($^{5'}$AC$^{3'}$-linker-Val)

Forty milligrams of support **6** (functionalization 0.14 mequiv/g) were used for the ODN chain assembly on an automated DNA synthesizer with the phosphoramidite derivative of 2′-deoxyadenosine, followed by standard oxidation and detritylation. Final treatment with benzoylchloride in pyridine (1/1, v/v, 1 h, rt) assured the capping of the 5′-OH end; after exhaustive washings with pyridine, CH_2Cl_2, CH_3CN, Et_2O, support **7** was dried under reduced pressure and then treated with 80 μL (0.49 mmol) of $Et_3N{\cdot}3HF$ in 450 μL of THF for 18 h at rt. Resulting support **8**, after washings with THF, pyridine, CH_2Cl_2, CH_3CN, Et_2O, was dried under reduced pressure and successively treated with 50 mg of 6-*N*-(4-monomethoxytriphenylmethyl)aminohexyl-(*O*-2-cyanoethyl, *N*,*N*-diisopropyl)-phosphoramidite dissolved in 1 mL of the activator solution (0.1 M tetrazole in

anhydrous CH_3CN). After usual washings, a standard oxidation step was performed giving functionalized support **9**. The incorporation of the linker molecule resulted to be almost quantitative (99% with respect to the initial nucleoside loading), as monitored by spectroscopic measurements of the 4-monomethoxytrityl cation ($\lambda_{max} = 472$ nm, $\varepsilon = 46770\ cm^{-1}\ M^{-1}$) released by acidic treatment of weighed amounts of the resin. Support **9** was then treated with DCA (10% solution in CH_2Cl_2) till complete disappearance of yellow coloured eluates, then washed as above described. The obtained solid support **10** was finally reacted with *N*-α-Fmoc-L-valine (27 mg, 0.23 mmol), PyBop (120 mg, 0.23 mmol), HOBt (31 mg, 0.23 mmol) and DIEA (30 μl) in 1 mL of anhydrous DMF (2 h, rt) and, after the appropriate washings, successively treated with a 20% piperidine solution in DMF at rt for 45 min, affording **11**.

Synthesis of hybrid b ($5'$AC$3'$-linker-Val-Phe)

For the preparation of **b**, we used the same procedure as for **a**, followed by coupling with the suitably protected phenylalanine derivative; for 35 mg of functionalized support (0.005 mmol), 77 mg (0.23 mmol) of *N*-α-Fmoc-D-phenylalanine, 118 mg (0.23 mmol) of PyBop, 30 mg (0.23 mmol) of HOBt and 30 mL of DIEA were added to the support suspended in 1 mL of anhydrous DMF. After 2 h at rt the support was exhaustively washed with DMF and then left in contact with a 20% piperidine solution in DMF at rt for 45 min.

For both **a** and **b**, Kaiser tests, carried out on the derivatized support after the piperidine treatment, resulted to be positive, confirming the removal of the Fmoc group from the amino terminus.

Synthesis of oligomer c ($5'$CTG-CTA-GAG-ATT-TTT-AC$3'$-linker-Gly-Gly-His)

The assembly of eptadecamer $5'$CTG-CTA-GAG-ATT-TTT-AC$3'$ was performed on an automated DNA synthesizer following standard phosphoramidite procedure.[16] Fifty milligrams of functionalized support **6** were used for 16 coupling cycles, using 2′-deoxyribonucleosides 3′-phosphoramidite as building blocks in concentration of 40 mg/mL. Coupling efficiencies, checked by spectroscopic DMT test, resulted to be always superior to 98%. After 5′-end benzoylation and 3′-OH deprotection, performed as described above, coupling with 50 mg of the phosphoramidite linker, dissolved in 1 mL of the activator solution (0.1 M tetrazole in anhydrous CH_3CN) and successive standard oxidation gave desired support **9**. The incorporation of the linker molecule resulted to be almost quantitative (99% with respect to the initial nucleoside loading), as monitored by spectroscopic MMT test. DCA treatment was carried out on support **9** to deprotect the terminal amino function, followed by the peptide synthesis; *N*-α-Fmoc-glycine (75 mg for each coupling, 0.25 mmol) and *N*-α-Fmoc-im-trityl-D-histidine (150 mg, 0.25 mmol) were used as building blocks in conjuction with PyBop (130 mg, 0.25 mmol), HOBt (35 mg, 0.25 mmol) and DIEA (85 μL) dissolved in 1 mL of anhydrous DMF. After each coupling step, a capping procedure by addition of acetic anhydride in pyridine (1/1, v/v) was carried out on the resin. Fmoc removal was performed as reported before by piperidine treatment, followed by Kaiser tests. The insertion of the histidine residue as the last aminoacid could be quantitated by spectroscopic MMT test performed on dried and weighed amounts of the resin, obtaining a final functionalization of 0.077 mequiv/g (i.e., 55% of the initial nucleoside loading).

Deprotection, purification, and characterization of hybrids a–c

Deprotection and detachment from the solid supports of the synthesized hybrids were obtained by overnight treatment with 0.1 M NaOH solution at room temperature. The supernatant was filtered and the support washed with water. The combined filtrate and washings were neutralized at pH 7.0 with acetic acid, then concentrated under reduced pressure, redissolved in water, analyzed and purified by HPLC. Hybrids **a** and **b** were purified on a Partisil 10 SAX column (Whatman, 4.6×250 mm, 7 μm) eluted with linear gradients of 1 mM KH_2PO_4 (20% CH_3CN, pH 7.0) in H_2O in 30 min, flow 0.8 mL/min, showing the following retention times: **a** = 20.13 min; **b** = 32.67 min. 17-mer **c** was purified on a Nucleogen DEAE 60-7 Macherey-Nagel column (125×4.0 mm, 7 μm); buffer A: 20 mM K_2HPO_4 aq. solution, pH 7.0, containing 20% (v/v) CH_3CN; buffer B: 1 M KCl, 20 mM K_2HPO_4 aq. solution, pH 7.0, containing 20% (v/v) CH_3CN, using a linear gradient from 10% to 100% B in 20 min, flow 0.8 mL min^{-1} (retention time 16.70 min). The isolated oligomers were collected and successively desalted by gel filtration on a Sephadex G25 column eluted with H_2O.

By HPLC analysis on a Waters μBondapak™ RP18 analytical column (Millipore, 300×3.9 mm, 5 μm), the isolated hybrids resulted to be more than 98% pure. Using a linear gradient (from 0 to 50% in 30 min) of CH_3CN in 0.1 M aq triethylammonium bicarbonate buffer, pH 7.0 (flow 0.8 mL min^{-1}, detection at $\lambda = 260$ nm), they showed the following retention times: **a** 11.70 min; **b** 12.32 min; **c** 22.62 min (natural 17-mer retention time: 18.50 min).

The synthesized 17-mer **c** was characterized by MALDI-TOF MS: found $[M+H]^+ = 5606.31$; mass calculated 5606.83.

a: ^{1}H NMR (400 MHz, D_2O) δ 8.39 (s, 1H, H-2 adenine residue), 8.31 (s, 1H, H-8 adenine residue), 7.86 (d, 1H, H-6 cytosine residue), 6.51 and 6.37 (dd, 1H each, 2 H-1′), 5.93 (d, 1H, H-5 cytosine residue), 4.91 (m, 1H, H-3′; the signal relative to the other 3′-proton is submerged by the solvent), 4.43 (m, 2H, 2 H-4′), 4.25 (AB part of an ABX system, 2H, H_2-5′ 2′-deoxycytidine unit), 3.95 (m, 4H, H_2-5′ 2′-deoxyadenosine unit and CH_2O linker), 3.79 (d, 1H, α CH valine residue), 3.32 (m, 2H, CH_2NH linker), 2.91 and 2.61 (m, 2H each, H_2-2′ 2′-deoxycytidine and 2′-deoxyadenosine units), 2.25 (m, 1H, CH(CH_3)$_2$), 1.68 (t, 2H, OCH_2CH_2 linker), 1.58 (t, 2H, CH_2CH_2NH linker), 1.41 (m, 4H, 2 CH_2 linker),1.09

(dd, 6H, $CH(CH_3)_2$ valine residue). FABMS: m/z 817 $(M\text{-}H)^-$.

b: ^1H NMR (400 MHz, D_2O) δ 8.32 (s, 1H, H-2 adenine residue), 8.24 (s, 1H, H-8 adenine residue), 7.83 (d, 1H, H-6 cytosine residue), 7.73–7.49 (complex signals, 5H, aromatic protons phenylalanine residue), 6.45 and 6.34 (dd, 1H each, 2 H-1′); 5.92 (d, 1H, H-5 cytosine residue), 4.98 (m, 1H, H-3′; the signals relative to the other 3′-proton and to α CH phenylalanine residue are submerged by the solvent), 4.41 (m, 2H, 2 H-4′), 4.20 (m, 3H, H_2-5′ 2′-deoxycytidine unit and α CH valine residue), 3.94 (m, 4H, H_2-5′ 2′-deoxyadenosine unit and CH_2O linker), 3.82 (m, 2H, CH_2Ph), 3.40 (t, 2H, CH_2NH linker), 2.83 and 2.59 (m, 2H each, H_2-2′ 2′-deoxycytidine and 2′-deoxyadenosine units), 2.34 (heptet, 1H, $CH(CH_3)_2$ valine residue), 1.68 (m, 4H, 2 CH_2 linker), 1.46 (m, 4H, 2 CH_2 linker), 1.41 (d, 6H, $CH(CH_3)_2$ valine residue. FABMS: m/z 964 $(M\text{-}H)^-$.

Thermal denaturation experiments

The concentration of the synthesized ODNs was determined spectrophotometrically at λ = 260 nm and at 80 °C, using the following molar extinction coefficients for each base:[17] 15400 (A); 11700 (G); 7300 (C); 8800 (T) $cm^{-1}\,M^{-1}$.

A 100 mM NaCl, 10 mM NaH_2PO_4 solution at pH 7.0 was used for the melting experiments. Melting curves were recorded realizing a concentration of approximately 2 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 10 °C for 20 min. After thermal equilibration at 20 °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 20–80 °C. The temperatures relative to the duplex melting, reported in Table 1, were determined as the maxima of the first derivative of absorbance versus temperature plots.

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