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Solid phase synthesis of oligonucleotides tethered to oligo-glucose phosphate tails

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Abstract—Oligonucleotides conjugated at both 3′ and 5′-ends with glucose residues, 4,6-linked through a phosphodiester bridge, have been synthesized by sequential addition of a 6-*O*-DMT-glucose-4-phosphoramidite building block following a standard automated ODN assembly procedure. Two 3′,5′-bis-glycoconjugated 18-mers, designed for antisense experiments, have been prepared and their hybridization properties with a complementary DNA fragment evaluated by UV thermal analysis. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Since the pioneering work of Zamecnick and Stephenson in the early 80's,[1] the scientific community recognized the wide potential of synthetic oligodeoxyribonucleotides (ODNs) as new therapeutic agents, able to specifically and efficiently block exogenous genetic messages without damaging the normal functions of the hosting cell. Gene expression inhibition by ODNs of specific sequence can be achieved targeting mRNA (antisense strategy)[2] by formation of stable hybrid DNA–RNA duplexes, where the RNA strand is rapidly degraded by endogenous RNase H. Alternatively, synthetic ODNs of specific sequences can hybridize double stranded DNA tracts (antigene strategy)[3] through formation of triple helical complexes, thus directly interfering with transcription processes. Moreover, ODNs can show highly selective recognition properties for specific proteins as well, acting as aptamers,[4] thus offering an extremely efficient and versatile approach to intervene at any stage of a pathogenic genetic information transfer, as, for example, of a virus or of an oncogene.

The dramatically low cellular uptake of ODNs associated with their very short half-life in cells, due to rapid degradation by nucleases, generally renders 'natural' ODNs completely inactive in in vivo systems. To improve the pharmacological profile of oligonucleotides, various chemical modifications, either at the level of the heterocyclic bases or of the sugar-phosphate backbone of the ODN chain, have been extensively investigated.[5,6] The replacement of the normal 3′,5′-phosphodiester linkages or of the natural ribose moieties in ODNs with suitable mimics in many cases led to enhancements in terms of bioavailability and nuclease resistance. As a matter of fact, a large number of differently modified ODNs are in clinical trials[7,8] and an antisense 21-mer phosphorothioate ODN (ISIS-2922, Vitravene) has been recently approved as an antiviral agent.[9] However, severe modifications, involving dedicated, sometimes cumbersome synthetic protocols, in most cases limit the accessibility of modified ODNs to very specialized laboratories.

As a valid alternative to a major chemical modification of the native structure of ODNs, conjugation is currently largely exploited for in vivo applications of ODNs: this strategy involves the preservation of the original ODN backbone (and therefore of the affinity towards the natural target) to which, at one or both ends of the chain, are covalently attached molecules imparting peculiar functions, such as peptides, polyethylene glycol, intercalators, fluorescent labels, or hydrophobic molecules as steroids, fatty acids, long chain alcohols, fullerene derivatives, etc.[10–12] By masking one or both ends of the ODN with any sterically demanding residue, a high degree of in vivo stabilization is definitively achieved, since exonucleases—by far the most abundant nucleases in cells—require free 3′ or 5′-OH ends to show nucleolytic activity. On the other hand, in order to reach an effective ODN concentration in cells, a specific delivery strategy has to be addressed, for example by linking specific 'carriers', as RGD or nuclear localization signal (NLS) peptide sequences, cationic tails as polylysine residues, PEG units, nanoparticles or hydrophobic molecules, to the ODN chain.

Oligosaccharides can also be useful carriers, relying on specific recognition mechanisms based on sugar-binding

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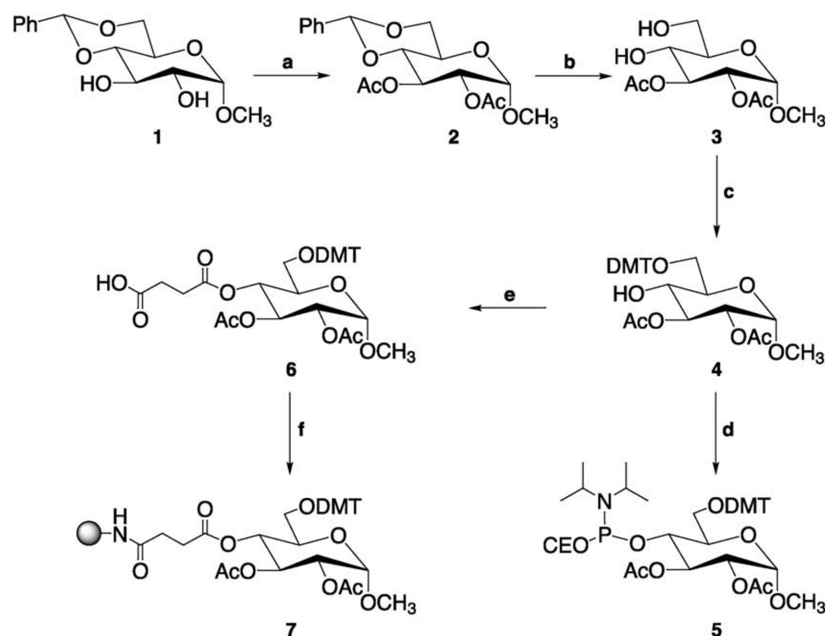


Scheme 1. (a) Ac_2O, pyridine, quantitative yield; (b) I_2, Et_3SiH cat., CH_3OH 70%; (c) DMTCl, TEA, DMAP cat., pyridine, 90%; (d) ClP(OCE)N(*i* Pr)$_2$, DIEA, DCM, 90%; (e) succinic anhydride, DMAP, pyridine, 92%; (f) CPG-NH_2, DCCI, pyridine.

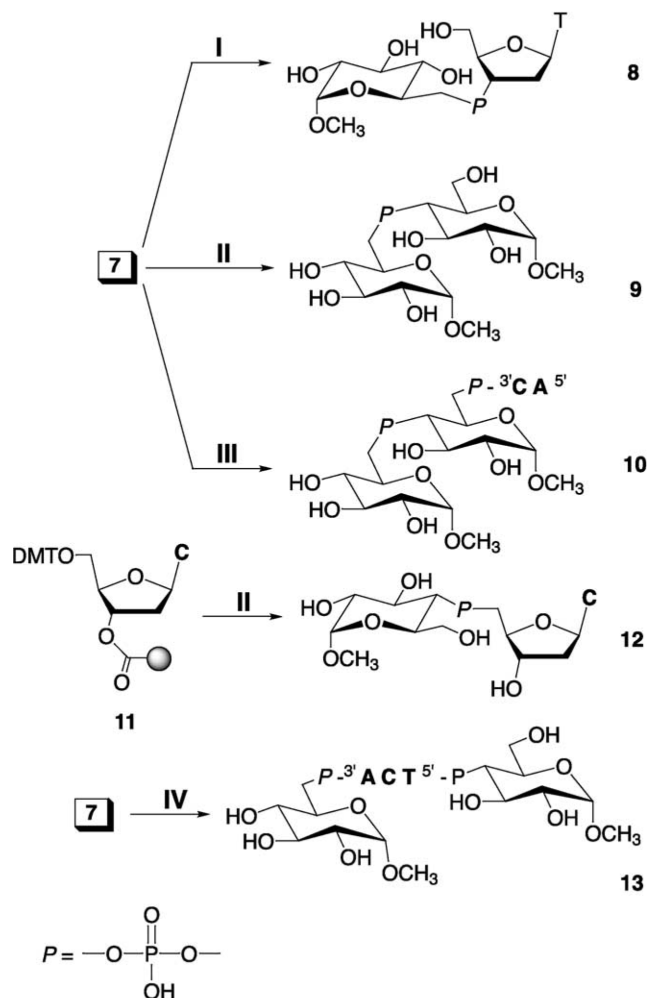
membrane receptors (lectins).[13] In this context, several protocols have been recently proposed to link a carbohydrate moiety to ODNs.[14–16] We recently reported[17,18] the first examples of direct, on-line glycosidation of the 5′-end of ODNs while still anchored to the CPG solid matrix and synthesized 3′,5′-bis-glucosylated oligomers starting from a new glucose functionalized support. However, this strategy is strictly limited to the insertion of a single sugar residue at the end of the ODN chains and its extension can hardly be envisaged to the synthesis of oligosaccharide–oligonucleotide conjugates. In fact, despite the notable efforts devoted to the solid phase synthesis of oligosaccharides,[19,20] the available methodologies are still far from being optimized so as to be successfully applied to a repetitive, high yielding oligomerization process. Further constraints are also present in this case, where the synthetic strategy for the assembly of the oligosaccharide portion must also be completely chemically compatible with the presynthesized DNA tract. In order to circumvent these limitations, mainly due to the glycosidation step, suitable oligosaccharide mimics can therefore be fruitfully devised.

A very common procedure to conjugate a precious molecule to an ODN chain involves its conversion into the corresponding phosphoramidite derivative.[10] Indeed this strategy has also been exploited in the case of sugars[14] or of base-glycosylated nucleosides,[21–25] which have been incorporated on the solid phase in high yields by following the same efficient chemistry adopted for ODN assembly,[26] resulting in stable phosphodiester linkages between the conjugating molecule and the 5′ or the 3′-OH terminus of the ODN. A similar approach has also been recently proposed by Joyce and co-workers, who designed a new class of DNA–carbohydrate conjugates, called nucleo-glycoconjugates, based on the synthesis of three new carbohydrate phosphoramidite derivatives, inserted at the extremities or at defined, internal positions of the growing oligonucleotide chain by standard, automated ODN synthesis protocols.[27]

With the aim of extending this methodology to the synthesis of stable and easy-to-handle oligosaccharide surrogates as new conjugating molecules for ODNs, we describe herein the synthesis and utilization of new glucose phosphoramidite building block **5**. This molecule, bearing the DMT as a transient protecting group for the 6-OH function, and the 2-cyanoethyl, *N*,*N*-diisopropylamino-phosphoramidite group as the reactive centre linked to the 4-position, can be sequentially coupled on an insoluble matrix. The usage of **5** in connection with glucose-functionalized support **7**[17] has allowed us to successfully prepare 3′,5′-bis-glucose-phosphate ODNs conjugates.

2. Results and discussions

Glucose building block **5** contains all the typical functional groups required in nucleotide monomers for the standard solid phase ODN assembly. This allows the efficient, sequential addition of a defined number of glucose-phosphate residues tethered to an ODN sequence. Its synthesis (see Scheme 1) has been carried out in four steps and 57% overall yields, starting from methyl 4,6-*O*-benzylidene-α-D-glucopyranoside **1**, which was converted into peracetylated **2** in quantitative yields by treatment with Ac_2O/pyridine. Then neat removal of the benzylidene protecting group was achieved by reaction with I_2 and cat. Et_3SiH in CH_3OH, furnishing **3**.[28] Selective dimethoxytritylation of the primary hydroxy function, performed by reaction with DMTCl and cat. DMAP in pyridine/TEA, gave desired **4**. This was next phosphitylated by addition of 2-cyanoethyl, *N*,*N*-diisopropylamino-chlorophosphoramidite and DIEA in anhydrous DCM, leading to **5**. Support **7** was synthesized starting from intermediate **4**, which, after



Scheme 2. **I**: (a) 1% DCA in DCM; (b) coupling with T 3′-phosphoramidite building block; (c) 1% DCA in DCM; (d) NH_4OH, 55°C, overnight. **II**: (a); (b) coupling with **5**; (c); (d). **III**: (a); (b) three coupling cycles with **5**, dC and dA 3′-phosphoramidite building blocks, respectively; (c); (d). **IV**: (a); (b) four coupling cycles with dA, dC, T 3′-phosphoramidite building blocks and **5**, respectively; (c); (d).

succinylation of the 4-OH function giving **6**,[30] was then reacted with CPG-NH_2 solid support as previously reported,[17] leading to an average functionalization of 0.050 mequiv./g, evaluated by DMT test. The identity and purity of all the synthesized compounds have been confirmed by NMR (^{1}H, ^{13}C and ^{31}P) and mass spectral data.

To test the feasibility of the proposed synthetic strategy for the preparation of new ODN conjugates, glucose phosphoramidite **5** was exploited in the solid phase synthesis of small hybrids **8–10**, **12** and **13** which, after purification, have been fully characterized by ^{1}H, ^{31}P NMR and ESI MS techniques.

As described in Scheme 2, support **7** was first exploited in a standard coupling with a nucleoside phosphoramidite affording, after deprotection and detachment, **8** as a model compound for the 3′-conjugation.

As a test to study and optimize the extension of the glucose-phosphate tail, consisting of glucose residues 4,6-linked through phosphodiester bonds, **5** was then coupled to glucosylated support **7**, affording **9**. The combined use of **5** and support **7** allowed the preparation of 3′-diglucosylated dinucleotide **10**. In order to check the efficiency of coupling with the 5′-OH function of a nucleoside, **5** was then reacted with CPG-bound deoxycytidine **11**, leading to **12**. As a model compound for the 3′,5′-conjugation, 3′,5′-bisglucosylated trinucleotide **13** was also synthesized. Remarkably, in all the studied cases, phosphoramidite **5**, coupled following a standard, automated protocol to the 5′-end of a CPG-bound nucleoside (as for **12**), of a growing ODN chain (as for **13**) or of a 6-OH function of a solid phase-linked glucose (as for **9** and **10**) showed absolutely similar efficiencies as classical nucleoside phosphoramidites (>98% yields, as evaluated by spectroscopic DMT test).

On this basis, two 18-mers of sequence d($^{5'}$GCGTGCCTCCTCACTGGC$^{3'}$), carrying at both the 3′ and 5′-ends one or two glucose-phosphate residues (**14** and **15**, Fig. 1), have been synthesized starting from support **7**, following standard automated phosphoramidite procedures. This ODN sequence is complementary to a tract of the mRNA of protein kinase A type 1 (PKA1), subunit RIα, which is overexpressed in the majority of human cancers and well recognized as a relevant target for therapeutic intervention against neoplasia; therefore several modifications of this sequence have been designed for antisense experiments and are currently under clinical trial.[31]

Detachment from the solid support and deprotection of the conjugated oligomers were achieved by treatment with concentrated aq. ammonia. Purification was carried out by HPLC on an anionic exchange Nucleogel column; the HPLC profile of crude **15** is showed in Fig. 2.

The collected peaks were then desalted by gel filtration chromatography on a Sephadex G25 column eluted with H_2O. The isolated products were checked for purity by HPLC on an analytical RP18 column, showing them to be >98% pure. The synthesized compounds were

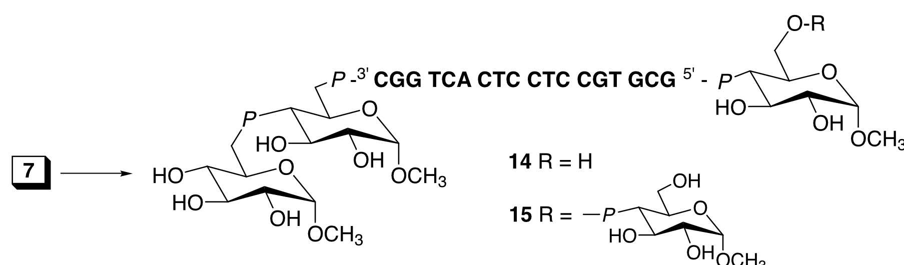


Figure 1. 3′,5′-Bis-glucoconjugates ODNs **14** and **15**.

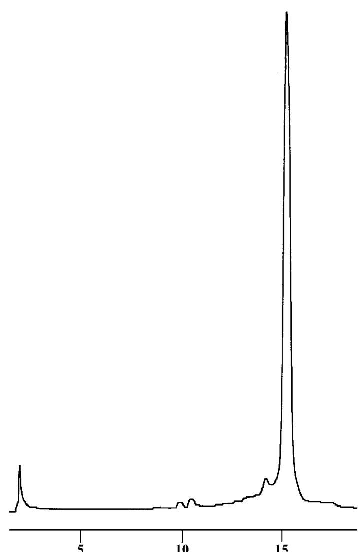


Figure 2. HPLC profile of crude **15** on a Nucleogel SAX column (see Section 4).

characterized by mass spectrometry; particularly, MALDI TOF MS spectra of each ODN conjugate showed a single peak in agreement with the calculated molecular weight.

In order to assess the influence of the glucosyl-phosphate tethers on the hybridization properties of the synthesized ODNs, thermal denaturation studies were carried out by mixing conjugates **14** or **15** with complementary 22-mer d($^{5'}$TTGCCAGTGAGGAGGCACGCAT$^{3'}$) in a 1:1 ratio in comparison with the same unmodified duplex. In both cases, the affinity towards the complementary sequence, expressed in terms of melting temperatures of the duplexes (T_m=60°C), showed not to be affected by the presence of the glucose-phosphate residues, thus demonstrating that this kind of conjugation does not negatively interfere with the recognition abilities of the ODN sequence. In renaturation experiments, the absorbance vs temperature profile was almost superimposable with the melting curve, showing the process to be, as expected, quasi-reversible.

The protecting effect of the glucose-phosphate residues on the synthesized ODNs towards nuclease activities was studied by incubating **14** with a 3′-exonuclease in a Tris–HCl buffer at 37°C. The unmodified 18-mer d($^{5'}$GCGTGCCTCCTCACTGGC$^{3'}$) was also incubated with the enzyme in the same experimental conditions for comparison. While the unmodified sequence was completely degraded within 2 h, glyco-conjugate **14** was totally unaffected even after 72 h. Experiments aimed at evaluating the antisense activity of the conjugated ODNs are currently underway in collaboration with specialized laboratories.

3. Conclusions

An efficient, on-line automated solid phase synthesis of a new class of ODNs conjugated to oligosaccharide mimics, easily obtained by sequential addition of novel DMT-glucose phosphoramidite **5**, has been described. The proposed method allows the elongation of ODNs with glucose-phosphate tails of the desired length by modulating the number of coupling cycles with building block **5**. This strategy offers several advantages: (i) **5** can be obtained by very straightforward and high yielding reactions; (ii) it can be coupled in extremely high yields following standard phosphoramidite protocols either to nucleoside-, oligonucleotide-, or sugar-immobilized OH functions; (iii) it can be exploited for several successive coupling cycles in an automated synthesis, since no apparent reduction in the efficiency of the coupling has been observed with increasing the number of glucose-phosphate residues. Several small hybrids have been synthesized and fully characterized to test the feasibility of this synthetic protocol. Two 3′,5′-conjugated 18-mers, complementary to a region of the mRNA of PKA1 (subunit RIα) which is a well studied antisense target, have been synthesized and characterized by MALDI TOF mass spectrometry. Experiments of duplex formation of **14** or **15** with a complementary DNA 22-mer, monitored by UV thermal denaturation analysis, showed that the presence of the oligo-glucose phosphate tails at the ends of the ODN chain did not impair its hybridization properties. In addition, as demonstrated by the experiment carried out on **14**, this modification renders the ODNs resistant to exonucleases.

Further studies are currently in progress to extend this methodology to the on-line solid phase synthesis of ODNs conjugated to phosphate-connected chains of other sugars or preformed oligosaccharides, previously converted into suitable phosphoramidite derivatives.

4. Experimental

4.1. Materials and methods

NMR spectra were recorded on Bruker WM-400 and Varian Gemini 200 and 300 spectrometers. All chemical shifts are expressed in ppm with respect to the residual solvent signal. ^{31}P NMR spectra were recorded on a Bruker WM-400 spectrometer using 85% H_3PO_4 as external standard. The solid support functionalizations were carried out in a short glass column (5 cm length, 1 cm i.d.) equipped with a sintered glass filter, a stopcock and a cap. The oligonucleotides were assembled on a Millipore Cyclone Plus DNA synthesizer, using commercially available 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl-phosphoramidite 2′-deoxyribonucleosides as building blocks.

The following abbreviations were used throughout the text: 4,4′-dimethoxytrityl (DMT), dichloromethane (DCM), diisopropylethylamine (DIEA), *N*,*N*-dicyclohexylcarbodiimide (DCCI), *N*,*N*-dimethylaminopyridine (DMAP), triethylamine (TEA).

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. MALDI TOF mass spectrometric analysis was performed on a PerSeptive Biosystems Voyager-De Pro MALDI mass spectrometer using a picolinic/3-hydroxypicolinic acids mixture as the matrix. For the ESI MS analysis a Waters Micromass ZQ instrument, equipped with an Electrospray source, was used in the negative mode.

Thermal denaturation experiments were carried out on a Jasco V-530 UV spectrophotometer with detection at λ= 260 nm and equipped with a Jasco ETC-505T temperature controller unit.

4.2. Synthesis of phosphoramidite 5

Compound **1** (564 mg, 2 mmol) was dissolved in pyridine (4 mL) and then acetic anhydride (2 mL) was added. After 2 h, CH_3OH was added, the mixture was diluted with DCM and the organic phase was washed three times with water. Concentration of the organic phase afforded pure **2** (732 mg, quantitative yield).

4.2.1. Compound 2. White solid, mp: 107–108.5°C (recrystallized from ethanol/water). R_f=0.75 (hexane–ethyl acetate 2:3, v/v). IR (KBr, cm^{-1}): 1748, 1460, 1379, 1235, 1200, 1146, 1126, 1093, 1061, 995, 963, 928, 775, 743, 703. ^1H NMR (300 MHz, $CDCl_3$) δ 7.20–7.45 (5H, m, aromatic protons); 5.56 (1H, t, $J_{2,3}=J_{3,4}$=9.8 Hz, H-3); 5.52 (1H, s, Ph-C*H*); 4.91 (1H, d, $J_{1,2}$=3.8 Hz, H-1); 4.88 (1H, dd, $J_{1,2}$=3.8 Hz, $J_{2,3}$=9.8 Hz, H-2); 4.30 (1H, dd, $J_{5,6eq}$= 3.7 Hz, $J_{6ax,6eq}$=9.8 Hz, H-6_{eq}); 3.92 (1H, m, H-5); 3.76 and 3.63 (1H each, two t's, H-4 and H-6_{ax}); 3.39 (3H, s, $-OCH_3$); 2.05 and 2.00 (3H each, s's, two $-COCH_3$). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.2 and 170.4 (*C*OCH_3); 136.6, 128.6, 127.8 and 125.8 (aromatic carbons); 101.1 (benzylidene CH); 97.1 (C-1); 78.7, 71.1, 68.6 and 68.3 (C-2, C-3, C-4 and C-5); 61.9 (C-6); 54.8 (1-OCH_3); 20.3 (CO*C*H_3). FAB MS (positive ions): *m/z* 367 $(M+H)^+$.

To a solution of **2** (732 mg, 2.0 mmol) in CH_3OH (11 mL) iodine (220 mg, 0.87 mmol) and triethylsilane (16 μL, 0.10 mmol) were added. After 3 h at room temperature the mixture was diluted with ethyl acetate and the organic phase washed with an aqueous solution of sodium bicarbonate and sodium thiosulfate. The organic phase was concentrated under reduced pressure and the residue was purified by silica gel chromatography (eluent DCM–CH_3OH from 98:2 to 95:5) to afford diol **3** (385 mg, 70% yield).

4.2.2. Compound 3. Colourless oil. R_f=0.45 (dichloromethane–methanol 9:1, v/v). ^1H NMR (200 MHz, $CDCl_3$) δ 5.30 (1H, t, $J_{2,3}=J_{3,4}$=9.8 Hz, H-3); 4.91 (1H, d, $J_{1,2}$= 3.8 Hz, H-1); 4.83 (1H, dd, $J_{1,2}$=3.8 Hz, $J_{2,3}$=9.8 Hz, H-2); 3.65–3.90 (4H, overlapped signals, H-4, H-5, H_2-6); 3.40 (3H, s, 1-OCH_3); 2.10 and 2.08 (3H each, s's, two $-COCH_3$). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.2 and 170.4 (*C*OCH_3); 96.6 (C-1); 72.6, 71.1, 70.8 and 68.6 (C-2, C-3, C-4 and C-5); 61.2 (C-6); 55.0 (1-OCH_3); 20.7 and 20.6 (CO*C*H_3). FAB MS (positive ions): *m/z* 279 $(M+H)^+$.

To a solution of compound **3** (372 mg, 1.3 mmol) and DMAP (9 mg, 0.07 mmol) in anhydrous pyridine (5 mL) 515 mg of 4,4′-dimethoxytriphenylmethyl chloride (1.5 mmol) and 207 μL of TEA (1.5 mmol) were added. The mixture was left under stirring overnight and then concentrated under reduced pressure. Silica gel chromatography of the residue (eluent petroleum ether–ethyl acetate 6:4 with few drops of TEA) afforded compound **4** (700 mg, 90% yield).

4.2.3. Compound 4. Oil. R_f=0.80 (hexane–ethyl acetate 2:3, v/v). ^1H NMR (200 MHz, $CDCl_3$) δ 6.83–7.58 (13H, m, aromatic protons); 5.30 (1H, t, $J_{2,3}=J_{3,4}$=9.7 Hz, H-3); 4.89 (1H, d, $J_{1,2}$=3.8 Hz, H-1); 4.83 (1H, dd, $J_{1,2}$=3.8 Hz, $J_{2,3}$=9.7 Hz, H-2); 3.79 (6H, bs, $-OCH_3$ DMT group); 3.62–3.76 (4H, overlapped signals, H-4, H-5, H_2-6); 3.39 (3H, s, 1-OCH_3); 2.75 (1H, d, *J*=2.8 Hz, 4-OH); 2.08 (6H, overlapped s's, two $-COCH_3$). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.2 and 170.4 (*C*OCH_3); 158.5, 135.7, 129.9, 128.0, 127.8, 126.7 and 113.1 (aromatic carbons DMT group); 96.5 (C-1); 86.4 (quaternary C DMT group); 72.8, 70.8, 70.8 and 69.8 (C-2, C-3, C-4 and C-5); 63.6 (C-6); 55.1 ($-OCH_3$ DMT group); 54.9 (1-OCH_3); 20.8 and 20.7 (two CO*C*H_3). FAB MS (positive ions): *m/z* 581 $(M+H)^+$; 303 $(DMT)^+$.

To a solution of compound **4** (637 mg, 1.1 mmol) in anhydrous DCM (7 mL) DIEA (580 μL, 3.3 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylamino-chlorophosphoramidite (380 μL, 1.7 mmol) were added at room temperature under argon. After 1 h the solution was diluted with ethyl acetate and the organic phase was washed with brine and then concentrated to afford a residue, which was then purified by silica gel chromatography (eluent petroleum ether–ethyl acetate 7:3 with few drops of TEA) to give **5** as a mixture of diastereoisomers (778 mg, 90% yield).

4.2.4. Compound 5. Glassy solid. R_f=0.55 (hexane–ethyl acetate 3:2, v/v). ^1H NMR (400 MHz, $CDCl_3$) δ 6.85–7.61 (26H, m, aromatic protons); 5.48 (2H, overlapped t's, $J_{2,3}=J_{3,4}$=9.6 Hz, two H-3); 4.99 and 4.97 (2H, two d's, $J_{1,2}$=3.7 Hz, two H-1); 4.81 and 4.85 (2H, two dd's, two H-2); 3.67 (12H, overlapped s's, $-OCH_3$ of DMT group); 3.54 and 3.56 (3H each, two s's, two 1-OCH_3); 4.00–3.15 (16H, overlapped signals, H-4, H-5, H_2-6, $-OCH_2CH_2CN$ and two $CH(CH_3)_2$); 2.30 and 2.54 (4H, overlapped t's, *J*=9.8 and 9.8 Hz, two OCH_2CH_2CN); 2.01, 2.03, 2.06 and 2.09 (3H each, four s's, four $COCH_3$); 1.03, 1.01, 0.98, 0.92 (24H, four d's, J_{vic}=6.6 Hz, $CH(CH_3)_2$). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.0 and 169.6 (two *C*OCH_3); 158.1, 144.8, 136.0, 135.7, 129.9, 129.8, 127.9, 127.3, 126.3 and 112.6 (aromatic carbons DMT group); 117.2 and 116.9 (CN); 96.1 (C-1); 85.5 and 85.4 (quaternary C of DMT group); 71.9, 71.1, 70.3 and 70.1 (C-2, C-3, C-4 and C-5); 63.2 and 62.5 (C-6); 58.4 and 58.0 (O*C*H_2CH_2CN); 54.7 and 54.6 (OCH_3 of DMT group and 1-OCH_3); 42.7 and 42.5 [*C*$H(CH_3)_2$]; 24.0 [$CH(CH_3)_2$]; 20.8 and 20.4 (two CO*C*H_3); 20.0, 19.9, 19.6 and 19.4 (OCH_2*C*H_2CN). ^{31}P NMR (161.98 MHz, $CDCl_3$) δ 152.1 and 151.0.

4.3. Functionalization of the resin. Support 7

Succinic anhydride (164 mg, 1.6 mmol) was added under argon to a solution of **4** (630 mg, 1.1 mmol) and DMAP (240 mg, 2.0 mmol) in anhydrous pyridine (5 mL). The mixture was left under stirring for 48 h at room temperature and then concentrated under reduced pressure. Silica gel chromatography of the residue (eluent dichloromethane–methanol 95:5, v/v) afforded pure **6** (680 mg, 92% yield).

Compound **6**:[30] glassy solid. R_f=0.19 (dichloromethane–methanol 9:1, v/v). ^1H NMR (200 MHz, $CDCl_3$) δ 6.82–7.55 (13H, m, aromatic protons); 5.45 (1H, t, $J_{2,3}=J_{3,4}$= 9.8 Hz, H-3); 5.09 (1H, dd, $J_{3,4}=J_{4,5}$=9.8 Hz, H-4); 5.00 (1H, d, $J_{1,2}$=3.6 Hz, H-1); 4.91 (1H, dd, $J_{1,2}$=3.6 Hz,

$J_{2,3}$=9.8 Hz, H-2); 3.91 (1H, m, H-5); 3.77 (6H, bs, $-OCH_3$ DMT group); 3.46 (3H, s, 1-OCH_3); 3.23 (2H, m, H_2-6); 2.10–2.50 (4H, m, succinic CH_2–CH_2); 2.08, 1.97 (3H each, s's, two $-COCH_3$). ^{13}C NMR (50 MHz) 174.6 ($-$*C*OOH); 170.6, 170.1 and 170.0 (two *C*OCH_3 and succinic ester CO); 158.2, 144.3, 137.1, 135.6, 129.8, 128.0, 127.5 and 126.5 (aromatic carbons); 96.3 (C-1); 85.7 (quaternary C of DMT group); 70.9, 69.9, 68.9 and 68.4 (C-2, C-3, C-4 and C-5); 61.7 (C-6); 54.9 (1-OCH_3 and $-OCH_3$ of DMT group); 28.7 and 28.6 (succinic CH_2); 20.4 ($COCH_3$). ESI MS (negative ions): *m/z* 679.28 $(M-H)^-$.

Compound **6** (305 mg, 0.45 mmol) and DCCI (277 mg, 1.34 mmol) were dissolved in anhydrous pyridine (10 mL). After 3 min the mixture was added under argon to a flask containing the CPG resin (300 mg). After 3 days under horizontal shaking, the resin was exhaustively washed with DCM and methanol and successively dried under reduced pressure. Spectroscopic DMT test on a weighed sample of dried support **7**[17] allowed the determination of the incorporation of the glucose derivative, which was 0.050 mequiv./g.

4.4. Synthesis of hybrids 8–10, 12 and 13. General procedure

50 mg of the starting support (**11** or **7**) were used for each synthesis. The coupling with **5** was performed on an automated DNA synthesizer following standard phosphoramidite chemistry,[26] using a 40 mg/mL solution of the amidite unit in anhydrous CH_3CN. Incorporation of the glucose phosphate residue within the chain always occurred with the same efficiency as for the usual nucleoside phosphoramidites, i.e. was always >98% yields (by DMT test). After completion of the desired ODN sequence and final DMT removal, the support was treated overnight with conc. aq. ammonia at 55°C. The filtered solution and the washings were concentrated under reduced pressure and purified by HPLC on a Nucleosil 100-5 C_{18} column (4.6×250 mm, 7 μm) eluted with a linear gradient of CH_3CN in TEAB buffer (0.1 M, pH 7.0). The final products were lyophilized and characterized by NMR and MS data. For compounds **10** and **13**, the reported mass is the calculated value on the basis of the combination of the multiply charged ions found.

4.4.1. Compound 8. ^1H NMR (400 MHz, D_2O), δ 7.65 (1H, s, H-6 T); 6.30 (1H, dd, *J*=6.0, 6.0 Hz, H-1′); 4.82 (1H, m, partially submerged by the residual solvent signal, H-3′); 4.76 (1H, d, *J*=4.0 Hz, H-1 Glu); 4.19 (1H, m, H-4′); 4.09 (2H, m, H_2-6 Glu adjacent to a phosphate residue); 3.80 (1H, dd, *J*=4.0, 10.0 Hz, H-2 Glu); 3.75 (1H, m, H-5 Glu); 3.67 (2H, m, H_2-5′); 3.63 (1H, d, *J*=10 Hz, H-3 Glu); 3.46 (1H, dd, *J*=10, 10 Hz, H-4 Glu); 3.39 (3H, s, OCH_3); 2.50 (2H, m, H_2-2′); 1.87 (3H, s, CH_3 T). ^{31}P NMR (161.98 MHz, D_2O), δ 1.42. ESI-MS: calculated mass: 498.13; found: *m/z* 497.14 $(M-H)^-$.

4.4.2. Compound 9. ^1H NMR (400 MHz, D_2O), δ 4.90 (2H, overlapped signals, 2H-1); H-4 Glu-phosphate is submerged by the residual solvent signal; 4.20 (2H, m, H_2-6 adjacent to a phosphate residue); 4.07–3.45 (8H, overlapped signals, two H-2, two H-3, two H-4, two H-5 and H_2-6OH); 3.48 and 3.47 (3H each, s's, 2OCH_3). ^{31}P NMR (161.98 MHz, D_2O), δ 2.01 ESI-MS: calculated mass: 449.11; found: *m/z* 448.61 $(M-H)^-$.

4.4.3. Compound 10. ^1H NMR (400 MHz, D_2O), δ 8.26 (1H, s, H-2 A); 8.18 (1H, s, H-8 A); 7.68 (1H, d, *J*=8.0 Hz, H-6 C); 6.36 and 6.23 (1H each, dd's, *J*=6.8, 6.8 Hz, 2H-1′); 5.75 (1H, d, *J*=8.0 Hz, H-5 C); 4.86 (2H, m's, partially submerged by the residual solvent signal, two H-3′); H-4 Glu-phosphate is submerged by the residual solvent signal; 4.70 (2H, overlapped signals, two H-1 Glu); 4.30 (2H, overlapped m's, two H-4′); 4.12–4.07 (6H, overlapped m's, H_2-5′ C and two H_2-6 Glu adjacent to a phosphate residue); 3.98–3.57 (8H, overlapped signals, H_2-5′ A, two H-2, two H-5 and two H-3); 3.46 (1H, dd, *J*=9.2, 9.2 Hz, H-4); 2.76 and 2.36 (2H each, m's, H_2-2′). ^{31}P NMR (161.98 MHz, D_2O), δ 1.53, 1.10, 0.68; ESI-MS: calculated mass: 1052.22; found: 1052.70.

4.4.4. Compound 12. ^1H NMR (400 MHz, D_2O), δ 8.02 (1H, d, *J*=7.6 Hz, H-6 C); 6.37 (1H, dd, *J*=6.4, 6.4 Hz, H-1′); 6.17 (1H, d, *J*=7.6 Hz, H-5 C); 4.88 (1H, d, *J*=4.0 Hz, H-1 Glu); H-4 Glu is submerged by the residual solvent signal; 4.60 (1H, m, H-3′); 4.30–4.18 (3H, overlapped signals, H-4′ and H_2-5′); 3.99 (1H, dd, *J*=8.0, 8.0 Hz, H-3 Glu); 3.88 (2H, m, H_2-6 Glu); 3.78 (1H, m, H-5 Glu); 3.66 (1H, dd, *J*=4.0, 8.0 Hz, H-2 Glu); 3.51 (3H, s, OCH_3); 2.45 (2H, m, H_2-2′). ^{31}P NMR (161.98 MHz, D_2O), δ 1.86 ESI-MS: calculated mass: 482.12; found: *m/z* 481.31 $(M-H)^-$.

4.4.5. Compound 13. ^1H NMR (400 MHz, D_2O), δ 8.54 (1H, s, H-2 A); 8.26 (1H, s, H-8 A); 7.72 (1H, s, H-6 T); 7.62 (1H, d, *J*=7.6 Hz, H-6 C); 6.53 (1H, dd, *J*=6.8 and 6.8 Hz, H-1′); 6.31 (1H, dd, *J*=7.2, 6.4 Hz, H-1′); 6.15 (1H, dd, *J*=7.2, 6.0 Hz, H-1′); 6.06 (1H, d, *J*=7.6 Hz, H-5 C); 5.15 and 4.91 (1H each, m's, two H-3′); 4.88 (1H, d, *J*=3.6 Hz, H-1 Glu); one H-3′, H-4 Glu phosphate and one H-1 Glu are submerged by the residual solvent signal; 4.55 and 4.39 (1H each, m's, two H-4′); 4.21 (9H, overlapped signals, one H-4′, three H_2-5′ and H_2-6 Glu adjacent to a phosphate residue); 4.06–3.56 (8H, overlapped signals, two H-2, two H-5, one H_2-6 Glu, two H-3 and one H-4); 2.96, 2.44 and 1.90 (2H each, m's, three H_2-2′); 1.99 (3H, s, CH_3 T). ^{31}P NMR (161.98 MHz, D_2O), δ 1.48, 1.32, 0.83, 0.58; ESI-MS: calculated mass: 1356.26; found 1356.23.

4.5. Synthesis of conjugates 14 and 15

ODN chain assembly was performed on 50 mg (25 μmol) of support **7** on an automated DNA synthesizer following a standard phosphoramidite protocol with final DMT removal.[26] Two sequences were assembled (**14** and **15**) inserting at the extremities of the growing sequence two or three coupling cycles with phosphoramidite **5**. The oligomers were detached from the support and deprotected by conc. ammonia treatment as described for hybrids **8–10**, **12** and **13**. The supernatant was filtered and the support washed with H_2O. The combined filtrates and washings were concentrated under reduced pressure, redissolved in H_2O and analyzed and purified by HPLC on a Nucleogel SAX column (Macherey-Nagel, 1000-8/46); buffer A: 20 mM KH_2PO_4 aq. solution, pH 7.0, containing 20%

(v/v) CH_3CN; buffer B 1 M KCl, 20 mM KH_2PO_4 aq. solution, pH 7.0, containing 20% (v/v) CH_3CN; a linear gradient from 0 to 100% B in 20 min, flow rate 0.8 mL/min, was used. The isolated oligomers, having the following retention times: **14**=15.17 min; **15**=16.85 min, were collected and successively desalted by gel filtration on a Sephadex G25 column eluted with H_2O. By HPLC analysis on a Nucleosil 100-5 C_{18} analytical column (4.6×250 mm, 7 μm), the isolated oligomers were >98% pure. Compound **14** MALDI TOF-MS: calculated mass: 6193; found: *m*/*z* 6192.10 $(M-H)^-$. Compound **15** MALDI TOF-MS: calculated mass: 6449; found: *m*/*z* 6447.22 $(M-H)^-$.

4.6. Thermal denaturation experiments

The concentration of the synthesized ODNs was determined spectrophotometrically at λ=260 nm and at 85°C, using the molar extinction coefficient calculated for the unstacked oligonucleotide using the following extinction coefficients: 15,400 (A); 11,700 (G); 7300 (C); 8800 (T) $cm^{-1}\ M^{-1}$.[32] For bis-conjugated oligomers **14** and **15**, the contribution of the glucose residues to the absorbance at λ=260 nm was considered negligible. A 100 mM NaCl, 10 mM NaH_2PO_4, aq solution at pH 7.0 was used for the melting experiments. Melting curves were recorded using 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 20–80°C. The melting temperatures, determined as the maxima of the first derivative of absorbance vs. temperature plots, are the average values of three independent melting experiments. For all the studied cases, both the modified duplexes and the unmodified one, within the experimental error (±0.5°C), exhibited a melting temperature value of 60°C.

4.7. Enzymatic experiments

3.0 OD of glycoconjugate **14** were incubated at 37°C with 1 unit of purified Exonuclease I (Amersham Biosciences, UK) in a 10 mM Tris–HCl, 50 mM KCl and 1.5 mM $MgCl_2$ buffer at pH 8.3. Aliquots of the mixture were taken at defined time intervals, kept at 80°C for 15 min, then left at room temperature and analyzed by HPLC on an analytical anion exchange column. A parallel experiment, carried out on 3.0 OD of unmodified ODN sequence d($^{5'}$GCGTGCCTCCTCACTGGC$^{3'}$), showed that the oligonucleotide was completely digested within 2 h. On the contrary, glycoconjugate **14** was totally stable even after 72 h.

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