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Synthesis and hybridization properties of inverse oligonucleotides

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ABSTRACT

The synthesis of adenine and thymine cyclopentylethyl nucleosides is presented. This novel constrained monomeric building block is very difficult to incorporate into oligonucleotides. It was introduced in 13mer oligodeoxynucleotide sequences at a single position using H-phosphonate chemistry. Phosphoramidite chemistry completely failed in this particular case. The H-phosphonate building blocks were obtained starting from the corresponding phosphoramidites. Stability of duplexes with RNA and DNA is significantly reduced.

INTRODUCTION

The synthesis of modified oligonucleotides is of great interest in the area of antisense therapeutics as well as for use as tools to study the functions of specific gene products in cells. As natural oligodeoxynucleotides cannot serve as antisense agents, an extensive search for modified substitutes has been carried out. Despite the huge numbers of modified analogues made so far, it is not possible to pinpoint the optimal chemical class of compounds for the desired therapeutic application. As even the most promising candidates suffer from certain drawbacks, there still is a need for new constructs. Previous studies conducted with acyclic nucleosides (1), pyranose oligonucleotides (2), 'bicyclo' oligonucleotides (3) and hexitol nucleic acids (4) have shown the importance of conformational pre-organization for the hybridization process. Natural nucleic acids have a ribofuranose–phosphate backbone which assures a certain flexibility by altering the puckering mode and through rotation around the phosphorylated CH_2OH functions. Here we present the synthesis and hybridization of 'inverse oligonucleotides', where the backbone consists of a phosphorylated cyclopentanediol moiety and the heterocyclic base is bound via a flexible ethylene linkage. Compared with the acyclic analogues with a 3(*S*)-dihydroxypentyl moiety (1), the cyclopentylethyl-modified single-stranded oligonucleotide has a more constrained carbocyclic backbone which may minimize entropy loss during duplex formation. Moreover, the ethylene linkage allows the base part to alter position, possibly enabling base pairing with natural nucleic acids (Fig. 1). These new oligonucleotides we name 'inverse oligonucleotides', as the five membered ring is now placed between the repeating phosphodiester bonds.

RESULTS AND DISCUSSION

The synthesis of the monomeric building block is performed starting from cyclopentanone **1** (Scheme 1). The hydroxyethyl dioxabicyclic intermediate **2** can be synthesized in 10 steps following a published procedure (5–7). The enantiomeric pure alcohol **2** is obtained through enzymatic resolution of the racemic ester by hog liver esterase, followed by $LiAlH_4$ reduction as described. The primary alcohol was then protected as its benzoyl derivative **3**. The enantiomeric purity of **3** was verified using capillary electrophoresis. Initial experiments using carboxymethylated β-cyclodextrin as a chiral electrolyte modifier showed that baseline separation of enantiomers could be obtained.

However, the minor optical component eluted after the major component and peak tailing prevented reliable determination of the enantiomeric excess. The additional inclusion of hydroxypropyl β-cyclodextrin in the electrolyte resulted in a reversed migration of the enantiomers, allowing improved determination of the minor component in the experimental samples. Typical electropherograms obtained under optimized conditions are shown in Figure 2 and illustrate that **3** could be considered as enantiomerically pure (<1% of the optical antipode).

Following deprotection of the isopropylidene group, deoxygenation of the hydroxyl group at the 2-position of **4** proved difficult. Several strategies were tried out and the most suitable proved to be a Barton-type reduction of the cyclic thiocarbonate **5**, yielding 31% of the desired 3-hydroxycyclopentyl derivative **6** and 45% of the 2-hydroxycyclopentyl congener. The resulting secondary hydroxyl group was protected as its dimethoxytrityl ether using the reactive dimethoxytrityl triflate (8). Alternative strategies, like selective protection of one of the hydroxyl groups of the diol functionality, either with a silyl moiety or directly with the DMTr group, followed by Barton-type reduction of the remaining secondary hydroxyl group, were not successful. The benzoyl group of **7** was removed by basic treatment and replaced with a tosyl moiety, affording **9**. The base moiety was introduced by nucleophilic substitution and the adenine derivative **10** was protected as its *n*-butylamino formamidine derivative (9) to obtain **12** (Scheme 2). Compound **9** was, likewise, used to synthesize the thymine derivative **11** using the lithium salt of the base.

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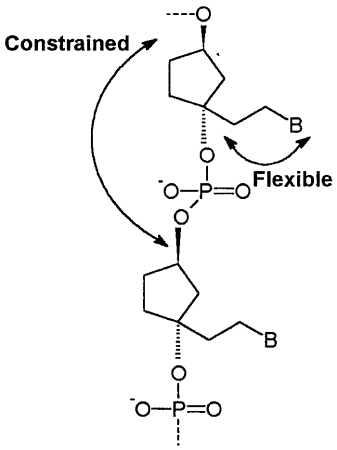


Figure 1. General structure of an 'inverse' oligonucleotide.

Synthesis of oligonucleotides

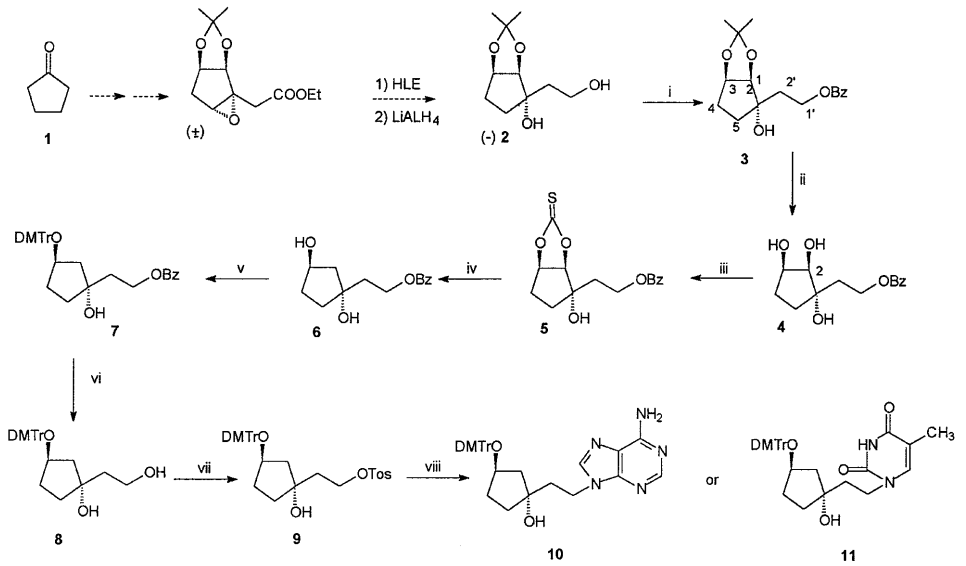
The protected cyclopentylethyl nucleosides **11** and **12** with respectively a thymine and an adenine base moiety were converted into their phosphoramidites as shown in Scheme 2. Assembly of oligonucleotides using these building blocks was attempted on an ABI 392 synthesizer at the 1 µmol scale using standard protocols, except for the use of 0.12 M amidites and 10 min coupling times. Coupling reactions using the cyclopentylethyl nucleotide building blocks (Scheme 2), however, failed completely. The reason for this is, at this time, not understood. Therefore, the phosphoramidites were hydrolysed and the cyanoethyl protecting group was removed by β-elimination, giving the H-phosphonates **15** and **16** (Scheme 3). These modified building blocks were used in an automated solid-phase synthesis using a combination of H-phosphonate and phosphoramidite chemistry as described by Seliger *et al.* (10). Following this procedure, the unmodified part of the oligonucleotide has been synthesized via standard phosphoramidite chemistry while the modified nucleotide was incorporated as the H-phosphonate. In this particular case the H-phosphonate chemistry seems to be superior to the phosphoramidite chemistry. The modified nucleotide could be successfully incorporated in 80% yield (as checked by a detritylation procedure). Correct incorporation of the modified nucleotide was proven by mass spectrometric analysis of the synthesized oligonucleotide.

Electrospray ionization (ESI) mass spectra (Fig. 3) showed the multiple charged species which, after deconvolution, gave the molecular weight. Only minor peaks were visible for the sodium adducts of the oligonucleotides.

Thermal stability of modified oligonucleotides

The synthesized oligonucleotides are shown in Tables 1 and 2.

The cyclopentylethyl nucleotides were incorporated into a homo-oligomer, affording $(dA)_6{\cdot}A^*(dA)_6$ and $(dT)_6{\cdot}T^*(dT)_6$ oligonucleotides, where A* and T* represent the modified nucleotide. The melting transitions demonstrate that although the modified adenine and thymine nucleotides maintain discriminatory capacity, hybridizing best with dT and dA respectively, stability of the duplexes is significantly decreased (ΔT_m of 4.3 and 11°C). The influence of the modified nucleotide on duplex stability was further investigated using a mixed sequence and the results were compared with those of the acyclic open chain analogues $A^{\neq}$ and $T^{\neq}$ (Table 2). Likewise, stability of the duplex containing natural nucleotides was higher than of duplexes containing the cyclopentylethyl nucleotides. The selectivity of base pairing within this sequence changed for the adenine derivative from AT > AG > AA > AC to A*G > A*T > A*A > A*C, as was also observed before



i) BzCl, pyridine; ii) HCl, H_2O/dioxane, r.t., total yield **2** to **4**: 85%; iii) $CS(Im)_2$, CH_3CN, 94%; iv) Bu_3SnH, AIBN, toluene, 31%; v) CF_3SO_2O(DMTr), pyridine; vi) NH_3/MeOH, total yield **6** to **8** 96%; vii) TsCl, pyridine, 100%; viii) B = Thymine, LiH, DMF, 60% or B = Adenine, LiH, DMF, 62%.

Scheme 1. Synthesis of dimethoxytritylated nucleoside analogs.

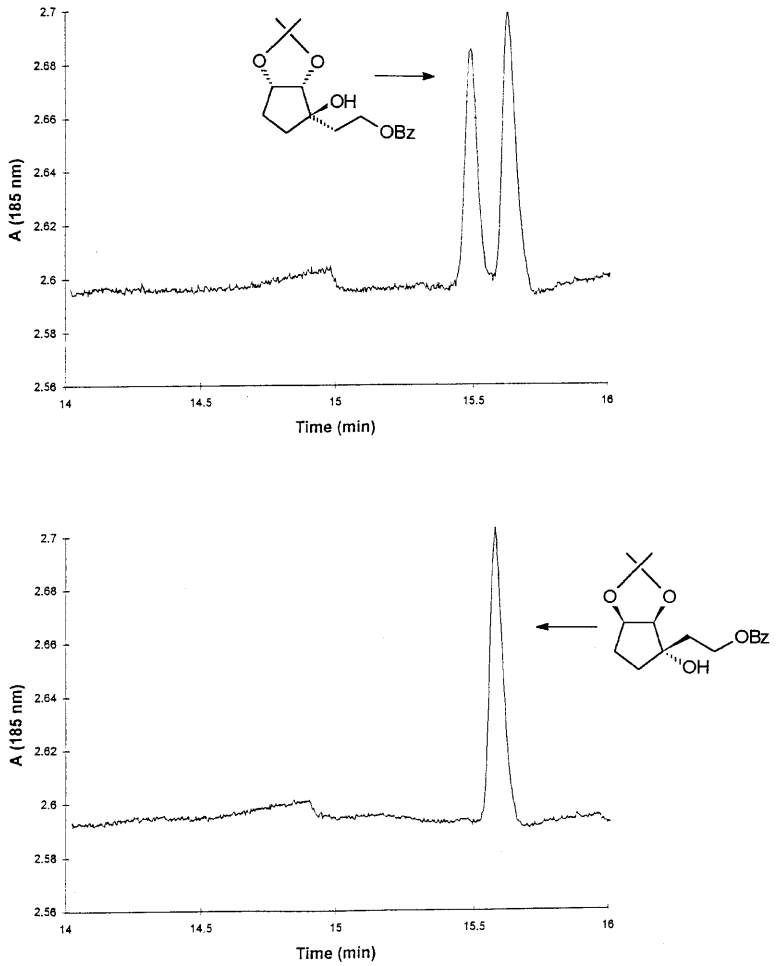
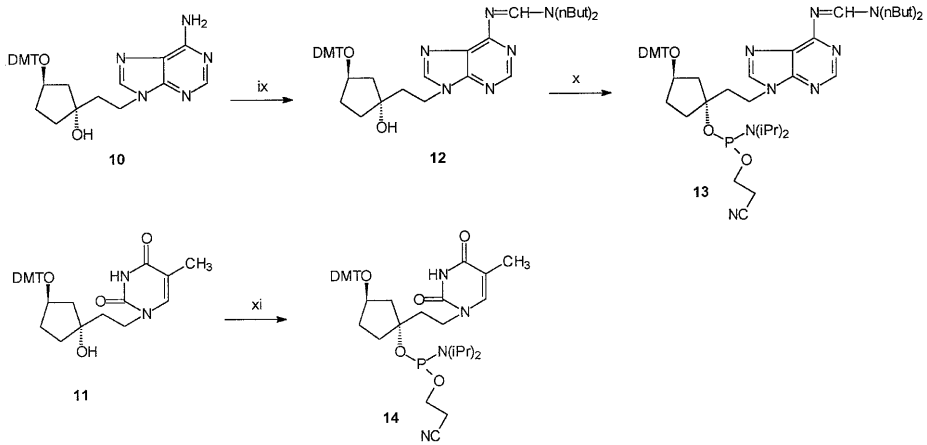
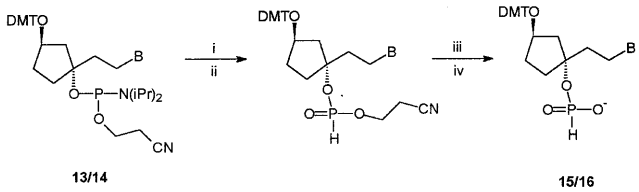


Figure 2. Analysis of enantiomeric purity of compound **3** using capillary electrophoresis and an electrolyte consisting of 10 mM MES containing 1% hydroxypropyl β-cyclodextrin and 1.5% carboxymethylated β-cyclodextrin polymer.



ix) N,N-Di-n-butylformamide dimethyl acetal, MeOH, 82%; x) $(iPr)_2NP(Cl)O(CH_2)_2CN$, $(iPr)_2NEt/CH_2Cl_2$, rt, 83%.; xi) same as x), yield 79%.

Scheme 2. Synthesis of phosphoramidite building blocks.



i) 0.5 M tetrazole in CH_3CN; ii) H_2O; iii) CH_3CN, DBU; iv) CH_3COOH conc.

13 and **15** B = N^6-(di-n-butylamino)methylene)-adenin-9-yl, **14** and **16** B = Thymin-1-yl.

Scheme 3. Synthesis of H-phosphonate building blocks from phosphoramidite precursors.

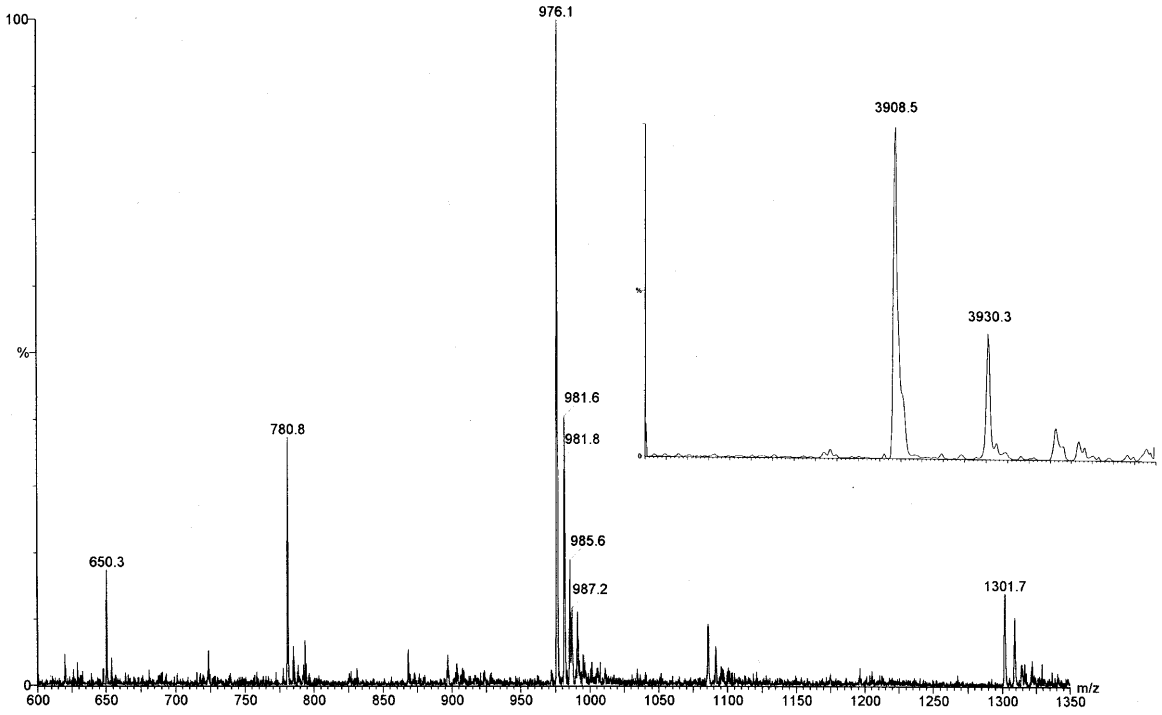


Figure 3. ESI mass spectrum of 5′-CAC CGT* CGG CGC C-3′ with the deconvoluted part of the molecular region in the inset.

with pyranose nucleosides (11) and to a lesser extent with the open chain acyclic nucleosides (1) (for structures see Fig. 4). This may reflect the preferential formation of purine–purine base pairs when the duplex is locally opened.

Where 'bicyclo' oligonucleotides suffered from too much constraint (3), we believed the acyclic oligonucleotides ($B^{\neq}$) to be too flexible (1). The hybrid structure proposed here (B*) was expected to overcome the disadvantages of the former two. Whereas improved hybridization was therefore expected for the newly described cyclopentylethyl nucleoside analogues B* in comparison with the open chain congeners $B^{\neq}$, the stability of all obtained duplexes containing inverse nucleoside analogues proved to be lower ($A^* < A^{\neq}$, $T^* < T^{\neq}$). Analogous results were obtained when evaluated versus a RNA complementary sequence, but determination of the melting temperature displayed a much broader curve.

Thermodynamic calculations were therefore carried out using the 'all or none' two state model developed by Gralla and Crothers (12; Table 3). Although only single incorporations have been accomplished, analysis of the obtained melting curves gives a possible explanation for the obtained results. For the mixed sequence oligonucleotide, as within an A_{13}-T_{13} homopolymer context, the enthalpy change is consistently lower for oligonucleotides containing an inverse nucleoside analogue (B*) compared with an acyclic one ($B^{\neq}$). The results obtained for the hybrid DNA–RNA duplexes confirm these observations.

Table 1. Melting temperatures (°C) of homo-oligomer duplexes
$d(T)_6\mathbf{X}d(T)_6$
$d(A)_6\mathbf{Y}(A)_6$

Y	X			
	G	C	A	T
A	20.0	17.9	18.5	34.6
A*	19.8	15.7	15.0	30.2
X	Y			
	G	C	A	T
T	21.0	20.7	34.6	21.3
T*	12.4	11.7	23.6	14.3

Table 2. Melting temperatures (°C) of hetero-oligomers containing the constraint nucleoside analogues with their complementary sequences

5′-GGCGCCG**Y**CGGTG-3′
3′-CCGCGGC**X**GCCAC-5′

X	Y			
	A	T	G	C
T	70.3	59.0	65.0	56.5
T*	62.7	59.1	60.1	54.7
T≠	64.6	59.6	60.7	55.6
A	61.5	70.0	67.0	58.3
A*	60.1	64.3	65.2	56.3
A≠	60.8	65.8	66.7	58.0

As expected, the loss in entropy upon duplex formation is likewise lower for oligonucleotides containing a pre-organized nucleoside analogue, but the difference is insufficient to compensate for the lower enthalpy contribution. The lack of hybridization capabilities therefore is mainly a consequence of reduced base pairing with the natural DNA chain, probably caused by insufficient positioning of the heterocyclic base opposite its complement and by rendering a more hydrophobic character to the duplex at the insertion site.

MATERIALS AND METHODS

Melting points were determined in capillary tubes with a Büchi-Tottoli apparatus and are uncorrected. UV spectra were recorded with a Philips PU 8700 UV/Vis spectrophotometer. The ^{1}H NMR and ^{13}C NMR spectra were determined with a Varian Gemini 200 MHz spectrometer with tetramethylsilane as internal standard for the ^{1}H NMR spectra and $CDCl_3$ (76.9 p.p.m.) for the ^{13}C NMR spectra. ^{31}P NMR spectra were obtained in $CDCl_3$ solution and chemical shifts are reported relative to 85% phosphoric acid/D_2O (external standard). (s, singlet; d, doublet; dd, doublet of doublet; t, triplet; br s, broad signal; m, multiplet.) Liquid secondary ion mass spectra (LSIMS) were obtained using a KRATOS Concept IH mass spectrometer. Samples were dissolved in 3-nitrobenzyl alcohol (nba), 2-nitrophenyl octyl ether (npoe), thioglycerol (Thgly) or thioglycerol doped with sodium acetate (Thgly-NaOAc). Electrospray ionization mass spectra were run on a VG Quattro II triple quadrupole system (Micromass, Manchester, UK). The oligonucleotide samples were prepared in an acetonitrile:water (1:1 v/v) mixture containing 0.01 M NH_4OAc. The final concentration of the oligonucleotides in the samples was ~20 pmol/µl. Capillary electrophoresis was performed on a Waters 4000 CE system (Waters, Milford, MA). Carboxymethylated β-cyclodextrin polymer and hydroxypropyl β-cyclodextrin were obtained from Cyclolab (Budapest, Hungary) and Amaizo (Hammond, IN) respectively. Other chemicals used were 4-[morpholino]ethane sulfonic acid (MES; Sigma, St Louis, MO), phosphoric acid (UCB, Leuven, Belgium) and HPLC grade methanol (Biosolve, The Netherlands). All compounds were used as received. Water used was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Column chromatography was performed on Janssen Chimica silica gel (0.060–0.200 nm). Anhydrous solvents were obtained as follows: dichloromethane, pyridine and acetonitrile were stored on calcium hydride, refluxed and distilled; tetrahydrofuran was refluxed overnight on lithium aluminium hydride and distilled. Melting temperatures were determined at 4 µM each strand in 0.1 M NaCl containing 0.02 M potassium phosphate, pH 7.5, and 0.1 mM EDTA. For thermodynamic calculations, absorbance values were sampled at a rate of 2 points/min with an increase in temperature of 0.2°C/min. The derivative at each point on the curve was determined by fitting a regression line to the point in a dynamically specified window containing 40 points (4°C). The transition enthalpy can be calculated from the equation $\Delta H = -18.28/(1/T_{½} - 1/T_{¾})$, as discussed in Loakes and Brown (13) and Habener *et al.* (14).

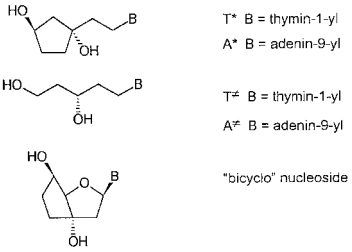


Figure 4. Comparison of the structure of a 1-alkyl-1,3-cyclopentanediol nucleoside with the structure of an acyclic nucleoside and a 3′,5′-ethanobridged nucleoside.

(1*S*,2*R*,3*R*)-1-(1′-Benzoyloxyethyl)-2,3-*O*-isopropylidene-1,2,3-cyclopentanetriol

Benzoyl chloride (0.19 ml, 1.6 mmol) was added dropwise to a solution of **2** (0.3 g, 1.5 mmol) in 15 ml dry pyridine at 0°C. The reaction mixture was stirred for 2 h at room temperature, evaporated, dissolved in CH_2Cl_2 and washed twice with saturated $NaHCO_3$ and brine. The organic layer was dried, evaporated and the resulting solid was used in the next step without further purification. A small sample was purified by column chromatography (hexane: EtOAc 7:1) for analytical purposes.

^{1}H NMR ($CDCl_3$) δ: 1.22, 1.41 (2 × s, 6H, 2 × Me); 1.54–1.95 and 2.0–2.33 [2 × m, 6H, CH_2(2′), CH_2(4), CH_2(5)]; 3.01 (br s, 1H, OH); 4.18 (dd, 1H, H-2, *J* = 1.3 and 5.5 Hz); 4.57 [t, 2H, CH_2(1′), *J* = 6.5 Hz]; 4.75 (t, H-3, *J* = 5.1 Hz), 7.35–7.6 and 8.01–8.05 (m, 5H, arom. H). ^{13}C NMR ($CDCl_3$) δ: 23.6 and 26.2 (2 × CH_3); 29.7 (C-4); 34.5 (C-2′); 35.1 (C-5); 61.9 (C-1′); 80.7 (C-3); 81.3 (C-1); 86.2 (C-2); 109.0 (C); 128.3, 129.4, 130.0, 130.1, 132.9 (aromatic C); 166.8 (CO). LSIMS (Thgly) exact mass calculated for $C_{17}H_{23}O_5$ $[M+H]^+$ 307.1545; found 307.1546.

(1*S*,2*R*,3*R*)-1-(1′-Benzoyloxyethyl)-1,2,3-cyclopentanediol (4)

Crude **3** (starting from 7.5 g, 37 mmol **2**) was dissolved in 500 ml 0.06 N HCl:dioxane (1:1) and stirred at 65°C for 3 h. The reaction mixture was cooled to room temperature, neutralized with solid $NaHCO_3$ and concentrated. The residue was extracted with CH_2Cl_2. Chromatographic purification on silica gel (1, CH_2Cl_2; 2, CH_2Cl_2/MeOH, 8:2) yielded 8.45 g (32 mmol, 86%) of a colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.52–1.75 and 1.9–2.3 [m, 6H, CH_2(4), CH_2(5), CH_2(2′)]; 2.74 (br s, OH); 3.01 (br s, OH); 3.45 (br s, OH); 3.75 (d, 1H, H-2), *J* = 4.5 Hz); 4.42–4.65 (m, 3H, CH2(1′), H-3); 7.37–7.60, 7.97–8.01 (m, 5H, aromatic H). ^{13}C NMR ($CDCl_3$) δ: 29.57 (C-4); 34.95 (C-2′); 35.26 (C-5); 62.08 (C-1′); 72.77 (C-3); 78.70 (C-2); 81.59 (C-1); 128.42, 129.5, 129.92, 133.13 (aromatic C); 167.12 (C=O). LSIMS (Thgly) exact mass calculated for $C_{14}H_{19}O_4$ $[M+H]^+$ 267.1232; found 267.1229.

Table 3. Melting temperature (°C) and thermodynamic data for the annealing of tridecadeoxynucleotide duplexes at 4 μM in 0.1 M NaCl

Duplex	T_m (°C)	$-\Delta H°$ (KJ/mol)	$-\Delta S°$ (K/mol K)	$-\Delta G°_{310}$ (KJ/mol)
3′-GTG GCT GCC GCG G-5′				
5′-CAC CG**A** CGG CGC C-3′	70.1	407.0	1076	73.4
5′-CAC CG**A***CGG CGC C-3′	64.3	377.2	1008	64.7
5′-CAC CG**A**≠CGG CGC C-3′	65.8	383.8	1023	66.8
3′-r(GUG GCU GCC GCG G)-5′[a]				
5′-CAC CG**A** CGG CGC C-3′	61.9	259.5	664.8	53.4
5′-CAC CG**A***CGG CGC C-3′	57.0	225.4	573.0	47.8
5′-CAC CG**A**≠CGG CGC C-3′	58.4	235.4	600.3	49.3
3′-GTG GCA GCC GCG G-5′				
5′-CAC CG**T** CGG CGC C-3′	70.0	402.2	1062	72.9
5′-CAC CG**T***CGG CGC C-3′	63.0	358.8	958	61.9
5′-CAC CG**T**≠CGG CGC C-3′	64.6	367.0	977	64.2
$d(T)_{13}/d(A)_{13}$	34.6	321.2	934	31.7
$d(T)_6$ **T*** $d(T)_6/d(A)_{13}$	23.6	294.5	883	20.9
$d(T)_6$ **T**≠ $d(T)_6/d(A)_{13}$	26.3	320.1	959	22.7
$d(A)_6$ **A*** $d(A)_6/d(T)_{13}$	30.2	318.5	940	27.0
$d(A)_6$ **A**≠ $d(A)_6/d(T)_{13}$	29.7	320.1	947	26.4

[a]A 22mer RNA complementary sequence was used with an overhang of five and four bases at the 3′- and 5′-end respectively: 3′-r(CGGGUGUGG-CUGCCGCGGGUGG)-5′.

(1*S*,2*R*,3*S*)-1-(1′-Benzoyloxyethyl)-2,3-*O*-thiocarbonyl-1,2,3-cyclopentanetriol (5)

Thiocarbonyl diimidazole (6.22 g, 34.8 mmol) was added to a solution of **4** (8.45 g, 31.7 mmol) in 250 ml dry CH_3CN. The reaction mixture was refluxed for 20 h at 120°C. After evaporation under reduced pressure, the residue was purified by column chromatography (hexane:EtOAc, 8:2–1:1), affording 8.8 g (94%) of a colourless oil.

^{1}H NMR ($CDCl_3$) δ: 1.68–2.13 and 2.15–2.4 [2 × m, 6H, CH_2(4), CH_2(5), CH_2(2′)]; 2.99 (br s, 1H, OH); 4.60 (t, 2H, CH_2(1′), *J* = 6.4 Hz); 4.86 (dd, 1H, H-2, *J* = 6.5 and 1.1 Hz); 5.43 (pseudo-t, 1H, H-3, *J* = 5.8 Hz); 7.41–7.59, 7.97–8.01 (m, 5H, aromatic H). ^{13}C NMR ($CDCl_3$) δ: 30.38 (C-4); 34.03 (C-2′); 34.60 (C-5); 61.10 (C-1); 87.57 (C-3); 90.23 (C-2); 128.56, 129.54, 133.45 (aromatic C); 167.19 (C=O); 190.96 (C=S). LSIMS (Thgly) exact mass calculated for $C_{15}H_{17}O_5S$ $[M+H]^+$ 309.0796; found 309.0803.

(1*S*,3*R*)-1-(1′-Benzoyloxyethyl)-1,3-cyclopentanediol (6)

A nitrogen purged solution of tri-*n*-butyltin hydride and AIBN in 75 ml anhydrous toluene was added dropwise to a refluxing solution of **5** (0.47 g, 1.6 mmol) dissolved in 70 ml anhydrous toluene. After 30 min the solvent was evaporated and the residue purified by column chromatography (hexane:EtOAc, 8:2–2:8), affording 110 mg (30%) of the title compound and 170 mg of the 2′-hydroxy derivative (43%).

^{1}H NMR ($CDCl_3$) δ: 1.5–1.85 and 1.9–2.3 [m, 8H, CH_2(2), CH_2(4), CH_2(5), CH_2(2′)]; 4.55 (pseudo-t, 3H, H-3, CH_2(1′), *J* = 6.2 Hz); 7.4–7.65, 8–8.04 (5H, aromatic H). ^{13}C NMR ($CDCl_3$) δ: 33.96 (C-4); 38.14 (C-5); 40.41 (C-2′); 49.86 (C-2); 62.08 (C-1′); 72.69 (C-3); 80.31 (C-1); 128.37, 129.47, 133.01 (aromatic C); 166.79 (C=O). LSIMS (Thgly) exact mass calculated for $C_{14}H_{19}O_4$ $[M+H]^+$ 251.1283; found 251.1274.

(1*S*,3*R*)-1-(1′-Benzoyloxyethyl)-3-*O*-(dimethoxytrityl)-1,3-cyclopentanediol (7)

Dimethoxytriphenylmethyl triflate (8) (0.55 g, 1.2 mmol) was added to a solution of **6** (0.25 g, 0.23 mmol) in 30 ml dry pyridine. The reaction mixture was stirred at room temperature overnight, quenched by addition of a saturated solution of $NaHCO_3$ and extracted with dichloromethane. The organic layer was dried and evaporated under reduced pressure. The residue was purified by column chromatography, affording 0.44 g of a colourless oil (83%).

^{1}H NMR ($CDCl_3$) δ: 1.10–1.92 [m, 6H, CH_2(2), CH_2(4), CH_2(5)]; 2.03 (t, 2H, CH_2(2′), *J* = 6.5 Hz); 3.77 (s, 6H, 2 × CH_3O); 4.16–4.34 (m, 1H, H-3); 4.45 (pseudo-t, 2H, CH_2(1′), *J* = 6.5 Hz); 6.75, 7.1–7.6, 8 (18H, m, aromatic H). ^{13}C NMR ($CDCl_3$) δ: 31.73 (C-4); 37.8 (C-2′); 39.88 (C-5); 48 (C-2); 55.19 (2 × CH_3O); 62.1 (C-1′); 74.61 (C-3); 79.61 (C-1); 86.39 ($Ph_3\underline{C}$); 113.06, 126.6, 127.7, 128.27, 129.52, 130.10, 133, 137.3, 145.98, 158.98 (aromatic C); 166.68 (C=O). LSIMS (Thgly-NaOAc) exact mass calculated for $C_{33}H_{36}O_6Na$ $[M+Na]^+$ 575.2409; found 575.2405.

(1*S*,3*R*)-1-(1′-Hydroxyethyl)-3-*O*-(dimethoxytrityl)-1,3-cyclopentanediol (8)

Compound **7** (0.24 g, 0.44 mmol) was dissolved in 100 ml methanolic ammonia and kept at room temperature for 2 days. After evaporation the residue was purified on a silica gel column eluting with hexane:EtOAc:Et_3N (30:70:0.5)/EtOAc (3:7) + 0.5% TEA, affording 0.190 g of a colourless oil (96%).

^{1}H NMR ($CDCl_3$) δ: 1.17–1.7 [m, 6H, CH_2(4), CH_2(5), CH_2(2)]; 1.78 (t, 2H, CH_2(2′), *J* = 6.5 Hz); 2.63 (2H, 2 × OH); 3.77[s, 2H, CH_2(1′)]; 3.81 (m, 6H, 2 × CH_3O); 4.18–4.3 (m, 1H, H-C); 6.78, 7.12–7.6, (13 H, aromatic H). ^{13}C NMR ($CDCl_3$) δ: 31.72 (C-4); 37.67 (C-2′); 41.75 (C-5); 47.59 (C-2); 55.18 (2 × CH_3O); 60.85 (C-1′); 74.52 (C-3); 81.60 (C-1); 86.34 ($Ph_3\underline{C}$); 113.04, 126.56, 127.72, 128.28, 130.093, 137.35, 145.99, 158.34 (aromatic C). LSIMS (Thgly-NaOAc) exact mass calculated for $C_{28}H_{32}O_5Na$ $[M+Na]^+$ 471.2147; found 471.2148.

(1*S*,3*R*)-1-(1′-*p*-Toluenesulfonyloxyethyl)-3-*O*-(dimethoxytrityl)-1,3-cyclopentanediol(9)

p-Toluenesulfonyl chloride (0.060 g, 0.31 mmol) was added to a solution of **8** (0.13 g, 0.23 mmol) in anhydrous pyridine and stirred at room temperature overnight. The reaction mixture was concentrated and the residue dissolved in dichloromethane and washed with saturated $NaHCO_3$ and brine. The organic phase was evaporated under reduced pressure and the residue was submitted to the following step without purification.

^{1}H NMR ($CDCl_3$) δ: 1.1–1.75 [m, 6H, CH_2(2), CH_2(4), CH_2(5)]; 1.91 (t, 2H, CH_2(2′), *J* = 6.6 Hz); 2.43 (s, CH_3); 3.7 (6H, 2 × CH_3O); 4.1–4.3 [m, 3H, H-3, CH_2(1′)]; 6.80 (d), 7.1–7.6 (17H, aromatic H). ^{13}C NMR ($CDCl_3$) δ: 21.64 (H_3C); 31.67 (C-4); 37.82 (C-2′); 40 (C-5); 47.88 (C-2); 55.20 (2 × CH_3O); 67.63 (C-1′); 74.41 (C-3); 79.14 (C-1); 86.95 ($Ph_3\underline{C}$); 113.08, 126.62, 127.75, 127.89, 128,21, 129.86, 130.09, 137.22, 144.13, 145.93, 147.45, 158.41 (aromatic C). LSIMS (Thgly-NaOAc) exact mass calculated for $C_{35}H_{38}O_7$ Na $[M+Na]^+$ 625.2236; found 625.2235.

(1*S*,3*R*)-1-[1′-(Adenin-9-yl)ethyl]-3-*O*-(dimethoxytrityl)-1,3-cyclopentanediol (10)

A mixture of adenine (0.43 g, 3.18 mmol) and LiH (24.3 mg, 6 mmol) in 2 ml dry DMF was stirred at 110°C for 30 min. A solution of the tosyl derivative **9** (0.7 g, 1.16 mmol) in 10 ml DMF was slowly added to this suspension. The reaction mixture was stirred at 110°C for 30 min, cooled to room temperature and evaporated under reduced pressure. The residue was dissolved in ethylacetate and washed with saturated $NaHCO_3$ and with brine. The organic phase was evaporated under reduced pressure and the residue purified by column chromatography (CH_2Cl_2 to CH_2Cl_2:5% MeOH in the presence of 0.5% pyridine), affording 0.4 g (62%) of a white foam. UV (MeOH) λ_{max} 262 nm (ε = 13 300).

^{1}H ($CDCl_3$) δ: 1.2–1.7 [m, 6H, CH_2(2′), CH_2(4′), CH_2(5′)]; 2.1 [m, CH_2(2′)]; 3.7 (6H, 2 × CH_3O); 4.1–4.4 [m, 3H, H-3, CH_2(1′)], 5.95 (br s, 2H, NH_2), 6.7–6.8, 7.15–7.5 (m, 13 H, aromatic H); 7.79 (s, 1H, H-2 of adenine); 8.3 (s, 1H, H-8 of adenine). ^{13}C NMR δ: 31.68 (C-4); 37.84 (C-5); 40.18 (C-1′); 41.49 (C-2′); 47.84 (C-2); 55.16 (CH_3O); 74.48 (C-3); 78.68 (C-1); 86.35 ($Ph_3\underline{C}$); 113.03, 126.59, 127.7, 128.2, 130.02, 137.19, 137.28, 158.34 (aromatic C); adenine C: 119.5 (C-5); 140.54 (C-8); 149.92 (C-4); 152.72 (C-2); 155.47 (C-6). LSIMS (Thgly-NaOAc) exact mass calculated for $C_{35}H_{38}O_7$Na $[M+Na]^+$ 588.2586; found 588.2607.

(1*S*,3*R*)-1-[1′-[*N*^{6}-((Di-*n*-butylamino)methylene)-adenin)-9-yl]-ethyl]-3′-*O*-(dimethoxytrityl)-1,3-cyclopentanediol (12)

A solution of **10** in 2 ml anhydrous MeOH was treated with *N*,*N*-di-*n*-butylformamide dimethyl acetal. The reaction mixture was stirred at room temperature for 4 h. After evaporation under reduced pressure the residue was purified by silica gel column chromatography eluting with CH_2Cl_2 to CH_2Cl_2:5% MeOH in the presence of 5% pyridine, affording 0.35 g (82%) of a white foam. UV (MeOH) λ_{max} 316 nm (ε = 23 000).

^{1}H NMR δ: 0.94 (t, 6H, 2 × CH_3); 1.16–1.77 (2 × m, 14H); 2.11 (t, 2H, *J* = 6.9 Hz); 3.39 (t, 2H, *J* = 7.33 Hz, N-C$\underline{H}_2$); 3.71 (t, 2H, *J* = 7.33 Hz, N-C$\underline{H}_2$); 3.7 (6H, 2 × CH_3O); 4.14–4.39 [2m, 3H, H-3, CH_2(1′)]; 6.7, 7.1–7.5 (13H, aromatic H); 7.85 (s, 1H), 8.5 (s, 1H); 9.0 (s, 1H). ^{13}C NMR δ: 13.88 and 13.65 (CH_3 of butyl); 20.17 and 20.19 ($\underline{C}H_2$ of butyl); 29.21 and 30.97 ($\underline{C}H_2$ of butyl); 31.68 (C-4); 37.74 (C-5); 39.99 (C-1′); 41.57 (C-2′); 45.18 (CH_2-N); 47.95 (C-2); 51.84 (CH_2-N); 55.14 ($H_3\underline{C}O$); 74.46 (C-3); 78.58 (C-1); 86.31 ($Ph_3\underline{C}$); 113.013, 125.95, 127.68, 128.20, 130.01, 137.18, 158.31, 145.84 (aromatic C); adenine C: 125.1 (C-5); 141.18 (C-8); 151.59 (C-4); 152.36 (C-2); 158.20 (C-6); 160.1 (N=$\underline{C}$H-). LSIMS (Thgly-NaOAc) exact mass calculated for $C_{35}H_{38}O_7$Na $[M+Na]^+$ 727.3947; found 727.3933.

(1*S*,3*R*)-1-[1′-[Thymin-1-yl)ethyl]-3-*O*-(dimethoxytrityl)-1,3-cyclopentanediol (11)

LiH (16.5 mg, 2 mmol) was added to a solution of thymine (0.27 g, 2 mmol) in 2 ml dry DMF. The suspension was stirred at 110°C for 30 min. The tosyl derivative **9**, dissolved in 10 ml DMF, was slowly added dropwise to this suspension. The reaction mixture was stirred at 110°C for 30 min and then cooled to room temperature. After evaporation under reduced pressure the residue was dissolved in EtOAc and washed with a saturated solution of $NaHCO_3$ and brine. The organic phase was evaporated under reduced pressure and the residue purified by column chromatography on SiO_2 (CH_2Cl_2 to CH_2Cl_2:5% MeOH in the presence of 5% pyridine), affording 0.35 g (60%) of a white foam. UV (MeOH) λ_{max} 275 nm (ε = 8500).

^{1}H NMR ($CDCl_3$) δ: 1.2–2.0 [m, 11H, CH_2(2), CH_2(4), CH_2(5), CH_2(2′), CH_3]; 3.77 [m, 8H, CH_3O, CH_2(1′)]; 4.18–4.3 (m, 1H, H-3); 6.79–6.99 (m, 4H, aromatic H); 6.94 (s, 1H, H-6 of thymine); 7.13–7.46 (m, 9H, aromatic H); 8.55 (s,1H, H-N). ^{13}C NMR ($CDCl_3$) δ: 12.29 (H_3C); 31.68 (C-4); 37.89(C-5); 40.50 (C-1′); 44.85 (C-2′); 47.64 (C-2); 55.24 (H_3CO); 74.63 (C-3); 78.65 (C-1); 86.84 ($Ph_3\underline{C}$); 113.13, 126.61, 127.75, 128.12, 130.27, 137.17, 137.3, 140.25, 145.97, 158.34 (aromatic C); thymine C: 111.13 (C-5); 137.17 (C-6); 151.14 (C-2); 163.44 (C-4). LSIMS (Thgly-NaOAc) exact mass calculated for $C_{33}H_{36}O_6$Na $[M+Na]^+$ 579.2471; found 579.2471.

General procedure for synthesis of the phosphoramidite derivatives

A mixture of the protected nucleosides **11** and **12**, 3 equiv. dry *N*,*N*-diisopropylethylamine and 1.5 equiv. cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in 3 ml dry dichloromethane was stirred at room temperature for 2 h. After addition of 1 ml EtOH and further stirring for 25 min, the mixture was poured into 25 ml dichloromethane and washed with a 5% aqueous $NaHCO_3$ solution and with a saturated NaCl solution, dried and evaporated. Column chromatography on silica gel with *n*-hexane:acetone:Et_3N as eluent afforded the amidite as a foam which was dissolved in a minimal volume of dry dichloromethane and added dropwise to 100 ml cold (–50°C) *n*-hexane. The precipitate was isolated, washed with *n*-hexane, dried and used as such for DNA synthesis.

(1S,3R)-1-[1′-[N^6*-((Di-n-butylamino)methylene)adenin-9-yl]-ethyl]-3′-O-(dimethoxytrityl)-1,3-cyclopentanediol-1-cyanoethyl-diisopropyl phosphoramidite (13).* Yield 83%. ^{31}P NMR ($CDCl_3$) δ: 141.17, 141.56 (d). LSIMS (nba) $[M+H]^+$ 905.

(1S,3R)-1-[1′-[Thymin-1-yl)ethyl]-3′-O-(dimethoxytrityl)-1,3-cyclopentanediol-1-cyanoethyl diisopropylphosphoramidite (14). Yield 79%. ^{31}P NMR ($CDCl_3$) δ: 141.26, 141.59 (d). LSIMS (npoe) $[M–H]^-$ 755.

General procedure for synthesis of the H-phosphonate derivatives

To a solution of the phosphoramidite derivatives **15** and **16** in 5 ml dry acetonitrile was added a solution (0.5 M) of tetrazole in acetonitrile (15 equiv.) followed after 2 min by 4 ml water. After 15 min the reaction mixture was evaporated and than co-evaporated with dioxane. The resultant crude product was dissolved in dry acetonitrile and submitted to β elimination of the cyanoethyl moiety by addition of DBU (10 equiv.). After 15 min stirring at room temperature, the reaction mixture was neutralized with concentrated acetic acid, poured into dichloromethane and washed with TEAB. The organic phase was dried and concentrated under reduced pressure. Purification was performed on silica gel using a MeOH gradient in CH_3Cl_3:2% TEA as mobile phase.

(1S,3R)-1-[1′-[N^6-((Di-n-butylamino)methylene)adenin-9-yl]-ethyl]-3′-O-(dimethoxytrityl-1,3-cyclopentanediol-1-H-phosphonate (15). ^{1}H NMR ($CDCl_3$) δ: 0.9–0.99 (m, 6H, 2 × CH_3); 1.2–2.7 [m, 8H, CH_2(2), CH_2(4), CH_2(5), CH_2(2′)]; 3.42 (t, 2H, *J* = 7.3 Hz, H_2CN); 3.71 (t, 2H, *J* = 7.7 Hz, H_2CN); 3.78 (s, 6H, 2 × CH_3O); 4.22–4.62 [m, 3H, H-3, CH_2(1′)]; 5.28 (P-H); 6.7, 6.8, 7.1–7.4 (m, 10H, aromatic H); 8.04 (s, 1H, H-2); 8.38 (P-H, *J* = 620 Hz); 8.5 (s, 1H, H-8); 9.12 (s, H-C=N-). ^{13}C NMR ($CDCl_3$) δ: 13.63 and 13.85 (CH_3), 19.71 and 20.13 (CH_2 n-Bu), 29.18 and 30.84 (-$\underline{C}H_2$-CH_2-N-); 31.65 (C-4); 36.60 (C-5, d, *J* = 5.29 Hz); 40.2 (C-2′, d, *J* = 8.19 Hz); 45.29 (CH_2-N); 46.58 (C-2, d, *J* = 4.1 Hz); 52.05 (CH_2-N); 55.14 (CH_3O); 74.12 (C-3); 86.40 (C); 86.63 (C-1, d, *J* = 7.17 Hz); 125.12 (C-5); 113, 126.58, 127.72, 128.21, 130.04, 137.17 (aromatic C); 142.67 (C-8); 145.87 (aromatic C); 150.69 (C-4); 158.32 (aromatic C); 159.13 (N=$\underline{C}$H-N-). ^{31}P NMR ($CDCl_3$) δ: –1.69 (d), *J* = 616.43. LSIMS (Thgly) exact mass calculated for $C_{42}H_{52}O_6N_6P$ $[M–H]^-$ 767.3686; found 767.3676.

(1S,3R)-1-[1′-[Thymin-1-yl)ethyl]-3′-O-(dimethoxytrityl)-1,3-cyclopentanediol-1-H-phosphonate (16). ^{1}H NMR ($CDCl_3$) δ: 1.4–2.35 [m, 11H, CH_2(2), CH_2(4), CH_2(5), CH_2(2′), CH_3]; 3.8 (s, 6H, CH_3O); 3.92 [m, 2H, CH_2(1′)]; 5.41 (m, 1H, H-3); 6.8, 7.11–7.53 (m, 14H, aromatic H); 5.2 and 8.3 (d, 1H, *J* = 611 Hz, H-P). ^{13}C NMR ($CDCl_3$) δ:11.99 (CH_3); 31.30 (C-4); 36.4 (d, *J* = 3.9 Hz, C-5); 38.61 (C-1′); 44.31 (C-2′); 46.54 (d, *J* = 5.3 Hz, C-2); 54.98 (CH_3O); 74.06 (C-3); 86.16 (C-1); 86.32 (C); 109.91 (C-5 of thymine); 112.83, 126.35, 127.5, 128.04, 129.8 (aromatic C); 137.03 (C-6 of thymine); 145.7, 141.29, 137.14 (aromatic C); 150.79 (C-2); 158.11 (aromatic C); 164.36 (C-4). ^{31}P NMR ($CDCl_3$) δ: –1.87 (d), *J* = 614.6 Hz. LSIMS (Thgly-NaOAc) calculated for $C_{33}H_{36}N_2O_8PNa_2$ $[M–H+2Na]^+$ 665.2005; found 665.2038.

CE operation

Enantiomeric purity of **3** was verified by capillary electrophoresis using a Waters Quanta 4000 CE system (Waters, Milford, MA). Detection occurred with a fixed wavelength UV detector equipped with a mercury lamp and a 185 nm filter. The system was operated at room temperature (21°C) at a constant voltage of 30 kV using the normal polarity mode with detection towards the cathodic end of the capillary. A fused silica capillary of 50 μm internal diameter and 90 cm length was used. The column was stored overnight filled with water. Each day operation was started with a vacuum purge with 0.5 M NaOH followed by water. All runs were preceded by a 4 min purge with the electrolyte. Samples were introduced by gravity-induced siphoning (Δh = 10 cm, 60 s injection time). The electrolyte consisted of 10 mM MES containing 1% hydroxypropyl β-cyclodextrin and 1.5% carboxymethylated β-cyclodextrin polymer; the pH of the electrolyte was adjusted to 6.0 with 1 M H_3PO_4. The electrolyte was filtered and degassed immediately before use. Data were recorded using the Waters Baseline software.

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