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# Synthesis and Enzymatic Digestion of an RNA Nonamer in Both Enantiomeric Forms

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**Abstract**—The d- and l-RNA nonamers of the sequence r(GCUUCGGC)T have been synthesised for X-ray crystallographic purposes. In vitro digestion of the unnatural optical antipode by snake venom phosphodiesterase I takes place at an approximately 1800-fold slower rate than that of the natural d-nonamer. The digestion experiments showed—to our knowledge for the first time—that l-RNA can indeed be cleaved enzymatically when phosphodiesterase I from snake venom is used—as opposed to a number of cellular ribonucleases—which sheds an interesting light on the evolution and possibly structure/function relationship of venom versus cellular degradation enzymes. The broad substrate specificity of this enzyme could be taken advantage of to study and further optimise the resistance towards biodegradation of therapeutic l-RNA aptamers. 

## Introduction

RNA crystal structure solving gained much interest in the recent decade, but it remains impossible to predict whether a particular RNA sequence will crystallise orderly or not. One parameter that is influenced by crystal packing forces is the solid state molecular volume ($=V_{\text{unit cell}}/Z$). According to Wallach's rule,[1] racemic crystals of compounds in the low molecular weight regime ('small' molecules) may be more stable and on average somewhat more densely packed than their chiral counterparts.[2] Crystal packing of larger molecules such as oligonucleotides or proteins, which crystallise with great numbers of disordered water molecules, may not be driven by the same effects as small molecules. There is, to our knowledge, only one example in the literature where a middle-sized oligonucleotide was crystallised in both chiral and racemic forms, the naturally configured and the racemic crystal structures of the hexadeoxyribonucleotide d(CGCGCG). The solid state molecular volume of the duplex $d(CGCGCG)_2$ is 20% larger in the racemic[3] than in the chiral[4] crystal (7.54 versus 6.27 $nm^3$ per duplex) revealing a possible lack of relevance of Wallach's rule to highly solvated molecules. While 20% seems to be a significant difference, it could nevertheless be a fortuitous result, since the molecular volume may strongly depend on the quality of the solvent-rich crystal(s) actually measured. To obtain more information about the chiral-versus-racemic-difference in molecular volumes of middle-sized oligonucleotides is one reason for the present study.

Having successfully crystallised RNA in well diffracting single crystals, the next problem is to solve the possibly unprecedented diffraction data. The phases are usually solved by either molecular replacement (using similar model structures already solved) or by isomorphous replacement methods (using heavy atom derivatives). Both methods meet severe limitations in the case of middle-sized biomolecular (especially RNA) crystal data.[5–7] However, racemic compounds usually crystallise—provided that no conglomerates are formed—in centrosymmetric unit cells, a fact that largely delivers crystallographers from the phase problem.

The naturally configured d-nonamer r(GCUUCGGC)T has already been studied. Its crystallisation occurs in the presence of $Rh(NH_3)_6Cl_3$ and the molecular structure has been elucidated to 1.6 Å resolution.[8] Taking advantage of the knowledge of the crystallisation conditions and of the high quality of this crystal, we synthesised both enantiomers, the d- and the l-nonamers r(GCUUCGGC)T, and wish to crystallise d- and l-forms together using established crystallisation procedures, in order to solve the crystal structure of the racemate.

Here we report the synthesis of the l-monomers and the oligomers in both d and l forms, and we describe their characterisation by HPLC, MALDI-ToF MS and circular dichroism, as well as the digestion of both oligomers with snake venom phosphodiesterase I and alkaline phosphatase.

For the synthesis of the d-nonamer we used the new commercially available and high-yielding TOM® (2′-O-*t*riisopropylsily*l*ox*y*m*e*thyl) phosphoramidites from Xeragon,

*Keywords*: oligoribonucleotide; l-RNA; phase problem; phosphodiesterase I; snake venom.

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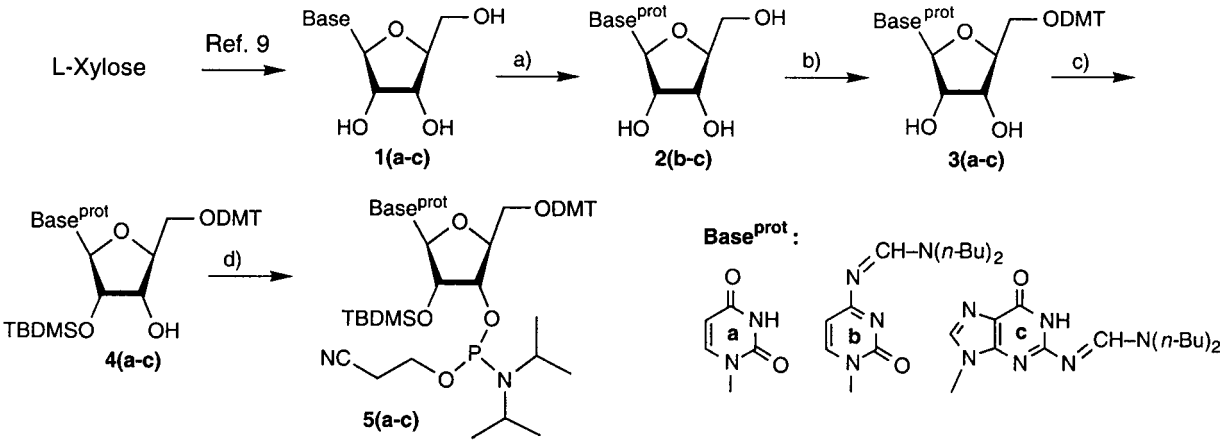



**Scheme 1.** Reaction conditions and isolated yields: (a) $(CH_3O)_2CHN(n\text{Bu})_2$, MeOH: (**2b**: 91%) (**2c**: 77%); (b) DMTCl, DMAP, pyr: (**3a**: 80%) (**3b**: 70%) (**3c**: 60%); (c) TBDMSCl, imidazole, pyr: (**4a**: 46%) (**4b**: 40%) (**4c**: 35%); (d) $ClP(Ni\text{Pr}_2)(OCH_2CH_2CN)$, 2,4,6-collidine, *N*-methylimidazole, THF: (**5a**: 88%) (**5b**: 73%) (**5c**: 91%).

Switzerland (purchased from Glen Research). The l-ribonucleoside phosphoramidites **5**(**a**–**c**) were prepared from the corresponding l-ribonucleosides **1**(**a**–**c**) previously synthesised from readily available l-xylose.[9] l-Thymidine derivative **15** was also synthesised to serve as the anchoring nucleoside for the RNA synthesis on solid support.

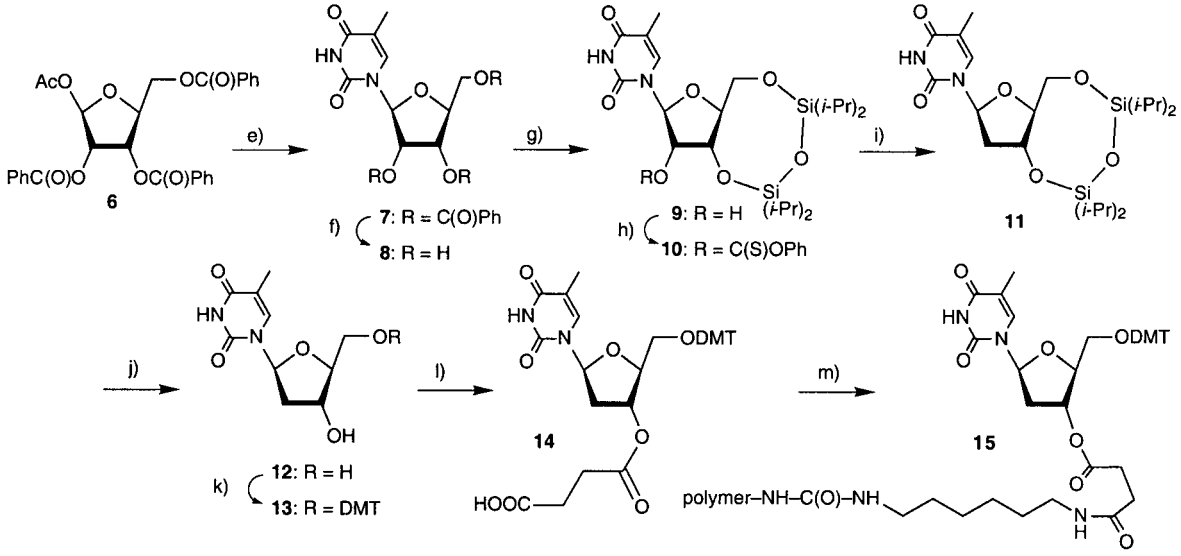
## Results and Discussion

### Synthesis of l-ribonucleoside phosphoramidites

We derivatised the l-ribonucleosides **1**(**a**–**c**) to the phosphoramidites **5**(**a**–**c**) according to a standard protocol (Scheme 1): (i) base amino protection, (ii) 5′-O-dimethoxytritylation, (iii) 2′-O-silylation, and (iv) 3′-O-phosphitylation. The *N*,*N*-di-*n*-butyl formamidine protecting group[10,11] was chosen because of the facile synthetic method, a simple one-flask procedure without any need of transiently protecting the hydroxyl groups. This amidine function is fully compatible with our oligoribonucleotide synthetic and deblocking protocol.

### Synthesis of the l-thymidine anchoring derivative

We elaborated the synthetic route on the D-series (not shown) and applied it to the l-anchoring derivative **15** (Scheme 2). Proceeding from 1-O-acetyl-2,3,5-tri-O-benzoyl-β-l-ribofuranose (**6**),[9] nucleoside derivative **7** was obtained in 98% yield through the well-established procedure reported by Vorbrüggen.[12,13] After two deprotection/reprotection steps (f, g), a slightly modified radical 2′-O-deoxygenation procedure according to Robins et al.[14] was applied on **9** (h, i) to furnish compound **11** in high yield. Further manipulations at the l-deoxyribose hydroxyl groups (j, k) afforded 5′-O-dimethoxytrityl derivative **13**. After coupling with succinic anhydride (l), compound **14** was




**Scheme 2.** Reaction conditions and isolated yields: (e) thymine, $CH_3C(=NHTMS)OTMS$; TMSOTf, $CH_3CN$, 65°C: 98%; (f) NaOMe/MeOH: 97%; (g) $(i\text{Pr}_2SiCl)_2O$, DMAP, pyr: 82%; (h) PhOC(S)Cl, DMAP, $CH_3CN$: 88%; (i) AIBN, $Bu_3SnH$, tol, 80–90°C: 82%; (j) $Bu_4NF$, THF: 75%; (k) DMTCl, DMAP, pyr: 81%; (l) succinic anhydride, $Et_3N$, DMAP, $(CH_2Cl)_2$: 87%; (m) hexamethylene diisocyanate/DMAP; $H_2NCH_2$–polystyrene (50% DVB), $EtN(i\text{Pr})_2$, $CH_2Cl_2$.

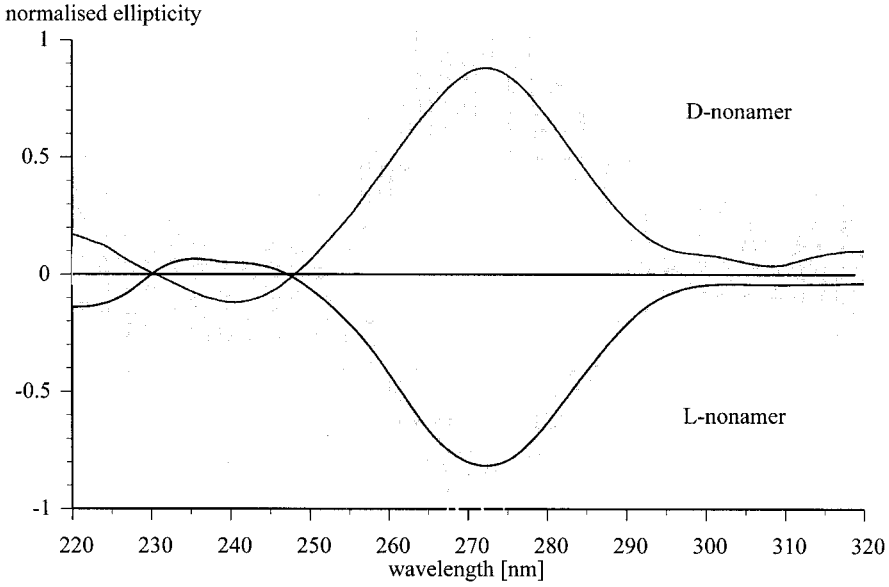



**Figure 1.** CD spectra of the d- and l-nonamers in aqueous buffer. Conditions: 50 μl nonamer stock solution (6 $OD_{260}$ L; 4 $OD_{260}$ D)+450 μl 100 mM NaCl, 10 mM $Na_XH_YPO_4$, pH 7.0.

loaded onto the solid support (aminomethyl polystyrene crosslinked with 50% divinyl benzene) as described by Kumar et al. (m).[15] A loading of 14 μmol **14**/g resin was obtained for the l-thymidine anchoring derivative and 17 μmol/g resin for the d-thymidine anchoring derivative.

### Synthesis of d- and l-oligoribonucleotides

The two oligomers were synthesised on an ABI 392 DNA/RNA synthesiser using a modified 1 μmol RNA cycle, 0.25 M 5-(benzylthio)tetrazole/$CH_3CN$, 10 equiv. of each l-ribonucleoside phosphoramidite and a coupling time of 600 s for the l-oligomer, 17 equiv. and 300 s for the d-oligomer, respectively. After deblocking with 40% aq. $CH_3NH_2$ (or 33% ethanolic $CH_3NH_2$) and $Et_3N{\cdot}3HF$, the oligoribonucleotides were precipitated from *n*-butanol at −20°C and purified by strong anion exchange HPLC, followed by desalting over $C_{18}$ reversed-phase using a step gradient from $H_2O$ to 30% aq. $CH_3CN$. The two HPLC-pure enantiomeric RNA nonamers were analysed by MALDI ToF mass spectroscopy and showed the expected molecular signals (HPLC and mass spectra in Experimental).

Circular dichroism (CD) spectroscopy of the two RNA nonamers resulted in spectra that were mirror images of each other giving physical evidence that the l-RNA nonamer was indeed the optical antipode of the corresponding d-RNA nonamer (Fig. 1).

### Digestion experiments

Both nonamers were submitted to an enzymatic digestion with snake venom phosphodiesterase I—a 5′-exonuclease that cleaves RNA and DNA strands from their 3′-terminus

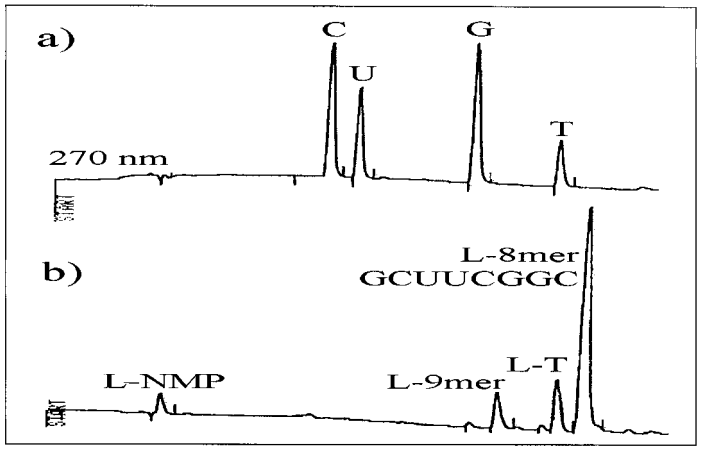



**Figure 2.** RP-HPLC analysis of the enzymatic digestion. (a) d-nonamer after 10 min at 37°C; area percent at 270 nm: 32.8% (13 min: 3×C), 20.3% (14.5 min: 2×U), 35.9% (20 min: 3×G), 11.3% (24 min: T). (b) l-nonamer after 20 min at 37°C. Digestion buffer: 12.5 mM Tris–OAc/12.5 mM $Mg(OAc)_2$, pH 8.8+3 milliunits snake venom phosphodiesterase I (5 units per mg, Merck) per pmol of RNA ($\epsilon_{260}$(9mer)=71200 $M^{-1}cm^{-1}$)+alkaline phosphatase (Sigma). HPLC conditions: Eurospher® 100/5 $C_{18}$ (Macherey–Nagel), 250×8 mm; A=0.1 M $NH_4OAc$, B=$CH_3CN$/ A 3:7; 0–20% B within 20 min; 2 ml/min; 270 nm. Intermediate nucleoside 5′-monophosphates (NMP) elute after approximately 5 min.

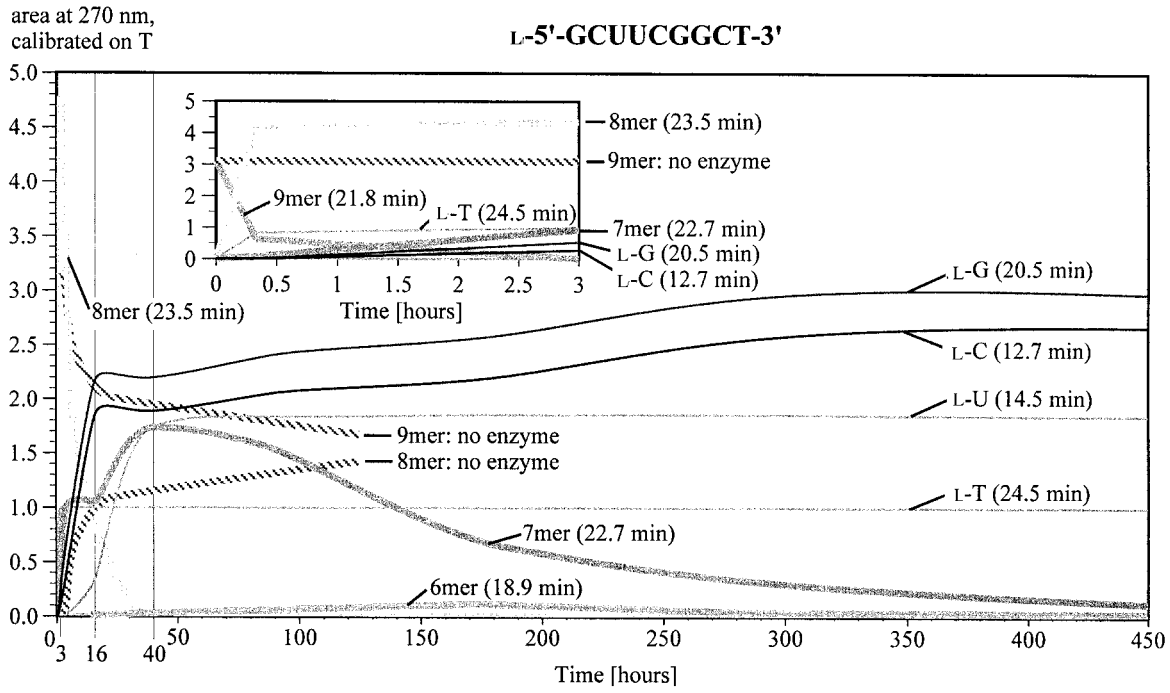



**Figure 3.** Time course of the digestion of the l-nonamer. Time course of the digestion of the l-nonamer at 37°C as analysed by RP-HPLC (conditions described in the legend of Fig. 2). Bold lines: degradation of l-oligomers; thin lines: built-up of l-nucleosides as observed at 270 nm (labels: identity and retention time of the HPLC signals). Time points taken after 20 min, 3 h, 16 h, 22 h, 40 h, 88 h, 124.5 h, 185 h, 305 h, and 477 h. The graphical insert shows the course during the first 3 h: rapid degradation of the l–9mer (dashed line: no degradation in the absence of enzymes), concomitant built-up of the l–8mer GCUUCGGC (maximum accumulation after 3 h) and l-thymidine (1 equiv. at 3 h), slower (but still relatively rapid) built-up of the l–7mer GCUUCGG, l-C and l-G. The main graph shows the appearance and disappearance of the observed signals within a time period of 450 h: 3 to 16 h: quite rapid accumulation of the l–7mer and the appearance of 2 equiv. of l-C and l-G each; 16–40 h: second accumulation of the l–7mer and almost full appearance of 2 equiv. of l-U, followed by a slow degradation of the l–7mer, the bearly visible intermediate accumulation of the l–6mer and the appearance the third equivalents of l-C and l-G.

releasing nucleoside 5′-monophosphates—and alkaline phosphatase (for complete dephosphorylation). As expected, the d-nonamer was completely degraded within 10 min at 37°C using 3 milliunits of phosphodiesterase I per pmol of nonamer. The d-ribonucleoside composition was in agreement with the primary sequence (Fig. 2a).

The assumed stereospecificity of the enzyme was expected to protect l-nucleic acids from nucleolytic cleavage, as shown by others that used whole cell extracts from mouse fibroblasts or RNase A,[16] a 3′-pyrimidine-specific endoribonuclease, nuclease $P_1$,[17] a 5′-nucleotidehydrolase, or human serum.[18,19] However, under identical conditions as for the d-nonamer (same enzyme/RNA ratio), the treatment of the l-nonamer showed the appearance of the octamer GCUUCGGC after only 20 min (Fig. 2b). After 3 h at 37°C, all l-thymidine was cleaved by the enzyme and other nucleosides (l-guanosine and l-cytidine) began to appear on the chromatogram. The time dependence of the digestion was followed by RP-HPLC until virtually complete (Fig. 3). Without enzymes but under otherwise identical conditions the l-nonamer was absolutely stable for at least 3 h, small amounts of the l-octamer GCUUCGGC began to appear after 6 h and steadily accumulated during the following 118.5 h (dashed lines in Fig. 3). No further degradation of the l-octamer was observed until enzyme was added after 124.5 h of incubation, when enzymatic digestion set in (not shown).

In conclusion, we synthesised an l-RNA nonamer utilising a useful nucleobase protecting group and an attractive workup protocol for the oligoribonucleotide. The digestion experiments showed—to our knowledge for the first time—that l-RNA can indeed be cleaved enzymatically when phosphodiesterase I from snake venom is used—as opposed to a number of cellular ribonucleases—which sheds an interesting light on the evolution and possibly structure/function relationship of venom versus cellular degradation enzymes. The rate of digestion of the l-RNA was at least some 1800-fold slower than that of the naturally configured d-RNA (≥300 h versus ≤10 min). In addition, the time course of the cleavage showed that (see also graphical abstract): (i) the thermally labile unpaired 3′-terminal l-deoxyribonucleoside was digested relatively fast (87% l-thymidine in 20 min), (ii) the rate of cleavage slowed down for the part involved in base pairing[20,21] (GCUUCGGC), and (iii) the cleavage of the 5′-terminal GC dinucleotide was the slowest (between approximately 50 and 500 h), which probably reflects uncatalysed RNA degradation owing to a continuing deactivation of the enzyme upon prolonged incubation at 37°.

## Experimental

### Abbreviations

AIBN, 2,2′-azobis[isobutyronitrile]; B, nucleobase fragment; BSA, N,O-*bis*-(trimethylsilyl)acetamide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; DMTCl, 4,4′-O-dimethoxyphenyl(phenyl)methyl chloride (= 4,4′-dimethoxytrityl chloride); hex: *n*-hexane; HMDIC,

hexamethylene diisocyanate; M, molecular ion; $R_f$, retention factor on silica tlc plates; PE, petrol ether; pyr, pyridine; TBDMSCl, *tert*-butyldimethylsilyl chloride; TBME, *tert*-butylmethyl ether; TCA, trichloroacetic acid; TEA, triethylamine; THF, tetrahydrofuran; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

## General

$^1$H NMR spectra were measured at 300 MHz on a VARIAN Gemini 300 spectrometer using tetramethylsilane ($\delta$=0.0) as an internal standard. $^{13}$C NMR spectra were obtained at 75 MHz using the signal from the solvents as internal standard ($CDCl_3$ ($\delta$=77.0), $CD_3OD$ ($\delta$=49.0)) and $^{31}$P NMR spectra using $POPh_3$ (as external standard ($\delta$=−18.0)). Mass spectra were obtained on a MAT 312 mass spectrometer using the Fast Atomic Bombardment (FAB) ionization method and positive ion detection (solvent: *p*-nitrobenzyl alcohol), or on a Perceptive Biosystems Vestec MALDI ToF mass spectrometer using 2,6-dihydrobenzophenone as the matrix ($N_2$ laser; neg. ion detection; 25 scans per spectrum). [$\alpha$] values were obtained on a Perkin–Elmer 141 polarimeter. Melting points were determined by visual observation on a Kofler block and are corrected. TLC was performed on a pre-coated silica gel $F_{254}$ plates with fluorescent indicator. Glycosides and nucleosides were visualised on tlc plates by subsequent spraying with naphtoresorcin and $H_2SO_4$ solutions in ethanol, respectively, followed by heating. Column chromatography was performed with flash silica gel (35–70 μm) from Uetikon; for the silylated compounds **4**(**a**–**c**): Silicagel H nach Stahl (Typ 60) from Merck; for the phosphoramidites **5**(**a**–**c**): Lichroprep Li 60 (40–64 μm) from Merck. Dry pyridine was obtained by distillation over $CaH_2$. All other solvents were used as purchased.

## Synthesis of l-ribonucleoside phosphoramidites

**6-*N*-((di-*n*-Butylamino)-methylene)-l-cytidine (2b).** l-Cytidine (**1b**) (74 mg, 0.30 mmol) was coevaporated three times in abs. pyr, then suspended in anhydrous methanol (0.7 ml). *N*,*N*-di-*n*-butylformamide dimethylacetal (105 mg, 0.52 mmol) was added and the mixture was stirred overnight at room temperature. After evaporation to dryness, purification by chromatography ($CH_2Cl_2$/MeOH 87:13) furnished **2b** (104 mg, 0.27 mmol, 91%) as a yellow solid. $R_f$ ($CH_2Cl_2$/MeOH 85:15): 0.58. $^1$H NMR ($CDCl_3$): 0.91–0.98 (m, 6H, $CH_3$); 1.26–1.40 (m, 4H, $CH_3CH_2$); 1.54–1.65 (m, 4H, $CH_3CH_2CH_2$); 3.32–3.55 (2m, 4H, $NCH_2$); 3.81 (dd, 1H, $^2J_{5'a,5'b}$=11.0 Hz, $^3J_{5'a,4'}$=3.2 Hz, HaC(5′)); 3.94 (dd, 1H, $^2J_{5'b,5'a}$=12.0 Hz, $^3J_{5'b,4'}$=4.4 Hz, HbC(5′)); 4.22–4.25 (m, 1H, HC(4′)); 4.31 (t, 1H, $^3J$=4.5 Hz, HC(3′)); 4.39 (t, 1H, $^3J$=4.9 Hz, HC(2′)); 5.69 (d, 1H, $^3J$=4.5 Hz, HC(1′)); 6.07 (d, 1H, $^3J$=7.2 Hz, HC(5)); 7.86 (d, 1H, $^3J$=7.2 Hz, HC(6)); 8.75 (s, 1H, NCH). $^{13}$C NMR ($CDCl_3$): 13.67, 13.76 ($CH_3$); 19.75, 20.06 ($CH_3CH_2$); 29.07, 30.93 ($CH_3CH_2CH_2$); 45.60, 52.29 ($NCH_2$); 62.03 (C(5′)); 70.82 (C(3′)); 75.28 (C(2′)); 86.08 (C(4′)); 94.11 (C(1′)); 103.34 (C(5)); 142.45 (C(6)); 157.42 (C(2)); 158.38 (NCH); 172.20 (C(4)). MS (FAB): 421 ([M+K]$^+$, 22.0%); 383 ([M+H]$^+$, 51.2%); 251 (100%).

**2-*N*-((di-*n*-Butylamino)-methylene)-l-guanosine (2c).** l-Guanosine (**1c**) (295 mg, 1.04 mmol) was treated as described above. Purification by chromatography ($CH_2Cl_2$/MeOH gradient from 90:10 to 85:15) gave **2c** (436 mg, 0.80 mmol, 77%) as a pale yellowish foam. $R_f$ ($CH_2Cl_2$/MeOH 85:15): 0.45. $^1$H NMR ($CD_3OD$): 0.94–1.01 (m, 6H, $CH_3$); 1.31–1.43 (m, 4H, $CH_3CH_2$); 1.60–1.70 (m, 4H, $CH_3CH_2CH_2$); 3.40–3.60 (2m, 4H, $NCH_2$); 3.74 (dd, 1H, $^2J_{5'b,5'a}$=12.2 Hz, $^3J_{5'a,4'}$=3.3 Hz, HaC(5′)); 3.84 (dd, 1H, $^2J_{5'b,5'a}$=12.1 Hz, $^3J_{5'b,4'}$=3.0 Hz, HbC(5′)); 4.09–4.12 (m, 1H, HC(4′)); 4.32 (dd, 1H, $^3J$=5.1, 3.5 Hz, HC(3′)); 4.66 (t, 1H, $^3J$=5.6 Hz, HC(2′)); 5.94 (d, 1H, $^3J$=5.9 Hz, HC(1′)); 8.06 (s, 1H, HC(8)); 8.65 (s, 1H, NCH). $^{13}$C NMR ($CD_3OD$): 14.05, 14.19 ($CH_3$); 20.76, 21.17 ($CH_3CH_2$); 30.17, 32.04 ($CH_3CH_2CH_2$); 46.70, 53.19 ($NCH_2$); 63.05 (C(5′)); 72.24 (C(3′)); 75.68 (C(2′)); 87.26 (C(4′)); 90.06 (C(1′)); 121.06 (C(5)); 139.39 (C(8)); 151.91 (C(4)); 159.35, 160.17 (C(6)) or (C(2)); 159.62 (NCH). MS (FAB): 461 ([M+K]$^+$, 63.4%); 423 ([M+H]$^+$, 78.8%); 291 ([$B^{N=CH(n\text{-}Bu)_2}$+H$_2$]$^+$, 100%).

**5′-O-(4,4′-Dimethoxytrityl)-l-uridine (3a).** l-Uridine (**1a**) (595 mg, 2.44 mmol) was coevaporated three times with abs. pyr, then pyr (10 ml) was added under an Ar atmosphere. DMAP (59 mg, 0.49 mmol) and DMTCl (908 mg, 2.68 mmol) were coevaporated two times with abs. pyr, then dissolved in pyr (10 ml) then added to the solution. The mixture was stirred overnight at room temperature. Methanol (2 ml) was added and stirred for 30 min. $CH_2Cl_2$ (200 ml) was added and washed with sat. $NaHCO_3$ solution (50 ml). After drying over $Na_2SO_4$ and evaporation, purification by chromatography (EtOAc/MeOH gradient from 100:0 to 90:10, 1% v/v $Et_3N$) afforded **3a** (1.07 g, 1.95 mmol, 80%) as a white foam. $R_f$ (MeOH/EtOAc 1:9): 0.70. $^1$H NMR ($CDCl_3$): 3.48 (dd, 1H, $^2J_{5'a,5'b}$=11.0 Hz, $^2J_{5'a,4'}$=2.5 Hz, HaC(5′)); 3.53 (dd, 1H, $^2J_{5'b,5'a}$=11.0 Hz, $^3J_{5'b,4'}$=1.9 Hz, HbC(5′)); 3.78 (s, 6H, $CH_3O$); 4.20 (m, 1H, HC(4′)); 4.33 (dd, 1H, $^3J$=5.0, 2.7 Hz, HC(2′)); 4.42 (d, 1H, $^3J$=5.7 Hz, HC(3′)); 5.37 (d, 1H, $^3J$=8.1 Hz, HC(5)); 5.89 (d, 1H, $^3J$=2.7 Hz, HC(1′)); 6.82–7.40 (m, 13H, Ph); 8.00 (d, 1H, $^3J$=8.2 Hz, HC(6)). $^{13}$C NMR ($CDCl_3$): 55.25 ($CH_3O$); 61.96 (C(5′)); 69.92 (C(3′)); 75.62 (C(2′)); 84.00 (C(4′)); 87.08 (*C*–Ph(Ph–$OCH_3$)$_2$); 90.80 (C(1′)); 102.22 (C(5)); 113.33–136.05, 144.35, 149.70, 158.72 (Ph); 140.34 (C(6)); 151.09 (C(2)); 163.60 (C(4)). MS (FAB): 585 ([M+K]$^+$, 1.5%); 547 ([M+H]$^+$, 1.3%) 303 ([C–Ph(Ph–$OCH_3$)$_2$]$^+$, 24.9%); 242 (100%).

**6-*N*-((di-*n*-Butylamino)-methylene)-5′-O-(4,4′-dimethoxytrityl)-l-cytidine (3b).** Compound **2b** (90 mg, 0.23 mmol) was treated as described above. Purification by chromatography ($CH_2Cl_2$/MeOH 98:2, 1% v/v pyr) afforded **3b** (109 mg, 0.16 mmol, 70%) as a white foam. $R_f$ ($CH_2Cl_2$/MeOH 90:10): 0.64. $^1$H NMR ($CDCl_3$): 0.92–0.96 (m, 6H, $CH_3$); 1.26–1.40 (m, 4H, $CH_3CH_2$); 1.54–1.64 (m, 4H, $CH_3CH_2CH_2$); 3.32–3.55 (2m, 6H, $CH_2N$, $H_2C(5')$); 3.77 (s, 6H, $CH_3O$); 4.30–4.33 (m, 3H, HC(2′), HC(3′), HC(4′)); 5.91 (d, 2H, HC(1′), HC(5)); 6.80–7.39 (m, 13H, Ph); 7.97 (d, 1H, $^3J$=7.2 Hz, HC(6)); 8.61 (br s, 1H, NH); 8.83 (s, 1H, NCH). $^{13}$C NMR ($CDCl_3$): 13.60, 13.68 ($CH_3$); 19.68, 19.97 ($CH_3CH_2$); 28.99, 30.87 ($CH_3CH_2CH_2$); 45.41, 52.09 ($NCH_2$); 55.10 ($CH_3O$); 62.76 (C(5′)); 71.44 (C(3′)); 76.80 (C(2′)); 85.09 (C(4′)); 86.64 (*C*–Ph($PhOCH_3$)$_2$); 92.44 (C(1′)); 113.12–140.91, 149.64, 158.25 (Ph);

144.25 (C(6)); 157.66 (C(2)); 158.46 (NCH); 172.08 (C(4)). MS (FAB): 723 ($[M+K]^+$, 7.5%); 685 ($[M+H]^+$, 9.9%); 303 ($[C-Ph(PhOCH_3)_2]^+$, 100%).

**2-*N*-((di-*n*-Butylamino)-methylene)-5′-O-(4,4′-dimethoxytrityl)-l-guanosine (3c).** Compound **2c** (406 mg, 0.75 mmol) was treated as described above. Purification by chromatography ($CH_2Cl_2$/MeOH gradient 98:2 to 95:5, 0.1% v/v pyr) gave **3c** (325 mg, 0.45 mmol, 60%) as a white foam. $R_f$ ($CH_2Cl_2$/MeOH 94:6): 0.38. $^1$H NMR ($CDCl_3$): 0.92–0.99 (m, 6H, $CH_3$); 1.26–1.40 (m, 4H, $CH_3CH_2$); 1.55–1.68 (m, 4H, $CH_3CH_2CH_2$); 3.30–3.73 (m, 6H, $CH_2N$, $H_2C(5')$); 3.75 (s, 6H, $CH_3O$); 4.28–4.30 (m, 1H, HC(4′)); 4.39–4.41 (m, 1H, HC(2′)); 4.64 (d, 1H, $^3J$=5.2 Hz, HC(3′)); 5.97 (d, 1H, $^3J$=5.8 Hz, HC(1′)); 6.76–7.39 (m, 13H, Ph); 7.52 (s, 1H, HC(8)); 8.43 (s, 1H, NH); 8.54 (s, 1H, NCH). $^{13}$C NMR ($CDCl_3$): 13.67, 13.78 ($CH_3$); 19.73, 20.15 ($CH_3CH_2$); 28.95, 30.92 ($CH_3CH_2CH_2$); 45.77, 52.43 ($CH_2N$); 55.17 ($CH_3O$); 64.03 (C(5′)); 71.66 (C(3′)); 74.80 (C(2′)); 84.15 (C(4′)); 86.40 (*C*–Ph(Ph–$OCH_3)_2$); 87.70 (C(1′)); 119.44 (C(5)); 113.16, 126.79–136.46, 158.02 (Ph); 144.69 (C(8)); 150.63 (C(4)); 156.84, 157.70 (C(6) or C(2)); 158.49 (NCH). MS (FAB): 763 ($[M+K]^+$, 13.8%); 725 ($[M+H]^+$, 13.1%); 303 ($[C-Ph(Ph-OCH_3)_2]^+$, 100%).

**2′/3′-O-*tert*-Butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-l-uridine (4a/a′).** Compound **3a** (169 mg, 0.31 mmol) and imidazole (84 mg, 1.24 mmol) were coevaporated three times with anhydrous pyr under Ar. Abs. pyr (1.6 ml) followed by TBDMSCl (51 mg, 0.34 mmol) were added. The mixture was stirred overnight at room temperature. After evaporation to dryness, $CH_2Cl_2$ (100 ml) was added washed by sat. $NaHCO_3$ solution (15 ml). After drying over $Na_2SO_4$ and evaporation, purification by chromatography (toluene/EtOAc gradient from 95:5 to 50:50 (85:15 to 82:18 for **4a**, 70:30 to 50:50 for **4a**′)) afforded **4a** (94 mg, 0.14 mmol, 46%) and **4a**′ (88 mg, 0.12 mmol, 43%) as a white foam. $R_f$ ($CHCl_3$/EtOAc 6:4): 0.64 (**4a**) 0.44 (**4a**′). $^1$H NMR ($CDCl_3$) (**4a**): 0.16 (s, 3H, $CH_3$); 0.19 (s, 3H, $CH_3$); 0.93 (s, 9H, *t*Bu); 2.58 (br s, 1H, HOC(3′)); 3.46–3.55 (m, 2H, $H_2C(5')$); 3.79 (s, 6H, $CH_3O$); 4.10–4.12 (m, 1H, HC(4′)); 4.33–4.36 (m, 2H, HC(2′), HC(3′)); 5.29 (d, 1H, $^3J$=8.2 Hz, HC(5)); 5.95 (d, 1H, $^3J$=3.0 Hz, HC(1′)); 6.81–7.40 (2m, 13H, Ph); 7.95 (d, 1H, $^3J$=8.1 Hz, HC(6)); 9.14 (br s, 1H, NH). $^{13}$C NMR ($CDCl_3$) (**4a**): −5.19, −4.65 (($CH_3)_2$Si); 18.02 (*C*($CH_3)_3$); 25.67 (C(*CH*$_3)_3$); 55.25 ($CH_3O$); 62.32 (C(5′)); 70.46 (C(3′)); 76.34 (C(2′)); 83.56 (C(4′)); 87.21 ((*C*–Ph(Ph–$OCH_3)_2$); 88.71 (C(1′)); 102.28 (C(5)); 113.32–135.17, 158.72, 158.75 (Ph); 140.25 (C(6)); 150.22 (C(2)); 163.18 (C(4)). $^1$H NMR ($CDCl_3$) (**4a**′): −0.03 (s, 3H, $CH_3$); 0.07 (s, 3H, $CH_3$); 0.0.85 (s, 9H, *t*Bu); 3.34–3.56 (m, 2H, $H_2C(5')$); 3.79 (s, 6H, $CH_3O$); 4.07–4.09 (m, 1H, HC(4′)); 4.17–4.20 (m, 2H, HC(2′)); 4.40 (t, 1H, $^3J$=5.4 Hz, HC(3′)); 5.40 (d, 1H, $^3J$=6.3 Hz, HC(5)); 5.98 (d, 1H, $^3J$=3.0 Hz, HC(1′)); 6.83–7.41 (2m, 13H, Ph); 7.88 (d, 1H, $^3J$=6.0 Hz, HC(6)). $^{13}$C NMR ($CDCl_3$) (**4a**′): −4.93, −4.89 (($CH_3)_2$Si); 17.89 (*C*($CH_3)_3$); 25.58 (C(*CH*$_3)_3$); 55.12 ($CH_3O$); 61.65 (C(5′)); 71.10 (C(3′)); 75.09 (C(2′)); 83.66 (C(4′)); 86.98 ((*C*–Ph(Ph–$OCH_3)_2$); 89.54 (C(1′)); 102.39 (C(5)); 113.17–135.06, 143.96, 158.86 (Ph); 140.11 (C(6)); 150.55 (C(2)); 163.33 (C(4)). MS (FAB): 699 ($[M+K]^+$, 5.3%); 661 ($[M+H]^+$, 1.7%); 303 ($[C-Ph(Ph-OCH_3)_2]^+$, 100%).

**6-*N*-((di-*n*-Butylamino)-methylene)-2′/3′-O-*tert*-butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-l-cytidine (4b/b′).** Compound **3b** (92 mg, 0.14 mmol) was treated as described above. Purification by chromatography (toluene/EtOAc gradient from 95:5 to 30:70 (80:20 to 75:25 **4b**, 40:60 to 30:70 **4b**′)) afforded **4b** (43 mg, 0.055 mmol, 40%) and **4b**′ (40 mg, 0.05 mmol, 37%) as a pale yellowish foam. $R_f$ ($CHCl_3$/EtOAc 6:4): 0.65 (**4b**), 0.32 (**4b**′). $^1$H NMR ($CDCl_3$) (**4b**): 0.19 (s, 3H, $CH_3$); 0.32 (s, 3H, $CH_3$); 0.90–0.99 (m, 15H, *t*Bu, $CH_3CH_2$); 1.26–1.38 (m, 4H, $CH_3CH_2$); 1.51–1.64 (m, 4H, $CH_3CH_2CH_2$); 2.44 (d, 1H, $^3J$=8.7 Hz, HOC(3′)); 3.29–3.58 (2m, 6H, $CH_2N$, $H_2C(5')$); 3.80 (s, 6H, $CH_3O$); 4.05–4.09 (m, 1H, HC(4′)); 4.28–4.32 (m, 2H, HC(2′), HC(3′)); 5.74 (d, 1H, $^3J$=7.2 Hz, HC(5)); 5.94 (d, 1H, $^3J$=1.2 Hz, HC(1′)); 6.73–7.45 (m, 13H, Ph); 8.07 (d, 1H, $^3J$=7.2 Hz, HC(6)); 8.86 (s, 1H, NCH). $^{13}$C NMR ($CDCl_3$) (**4b**): −5.50, −4.39 (($CH_3)_2$Si); 13.65, 13.74 ($CH_3$); 18.07 (*C*($CH_3)_3$); 19.76, 20.04 ($CH_3CH_2$); 25.86 ((*CH*$_3)_3$C); 29.06, 31.00 ($CH_3CH_2CH_2$); 45.40, 52.11 ($CH_2N$); 55.18 ($CH_3O$); 61.94 (C(5′)); 69.49 (C(3′)); 76.67 (C(2′)); 82.89 (C(4′)); 86.77 (*C*–Ph(Ph–$OCH_3)_2$); 90.52 (C(1′)); 113.08–141.28, 158.32 (Ph); 144.43 (C(6)); 156.25 (C(2)); 158.55 (NCH); 171.94 (C(4)). $^1$H NMR ($CDCl_3$) (**4b**′): −0.07 (s, 3H, $CH_3$); 0.04 (s, 3H, $CH_3$); 0.82 (s, 9H, *t*Bu); 0.88–0.97 (m, 6H, $CH_3CH_2$); 1.26–1.40 (m, 4H, $CH_3CH_2$); 1.52–1.64 (m, 4H, $CH_3CH_2CH_2$); 2.62 (t, 1H, $^3J$=7.3 Hz, HOC(2′)); 3.24–3.61 (2m, 6H, $CH_2N$, $H_2C(5')$); 3.79 (s, 6H, $CH_3O$); 4.12–4.17 (m, 2H, HC(2′), HC(4′)); 4.35 (t, 1H, $^3J$=5.5 Hz, HC(3′)); 5.83 (d, 1H, $^3J$=7.5 Hz, HC(5)); 6.03 (d, 1H, $^3J$=3.3 Hz, HC(1′)); 6.82–7.42 (m, 13H, Ph); 8.05 (d, 1H, $^3J$=7.5 Hz, HC(6)); 8.84 (s, 1H, NCH). $^{13}$C NMR ($CDCl_3$) (**4b**′): −5.01, −4.76 (($CH_3)_2$Si); 13.66, 13.96 ($CH_3$); 18.01 (*C*($CH_3)_3$); 19.76, 20.05 ($CH_3CH_2$); 25.65 ((*CH*$_3)_3$C); 29.06, 30.98 ($CH_3CH_2CH_2$); 45.39, 52.10 ($CH_2N$); 55.20 ($CH_3O$); 62.07 (C(5′)); 71.25 (C(3′)); 75.98 (C(2′)); 83.57 (C(4′)); 86.74 (*C*–Ph(Ph–$OCH_3)_2$); 91.18 (C(1′)); 113.19–141.29, 158.28 (Ph); 144.18 (C(6)); 156.75 (C(2)); 158.60 (NCH); 172.00 (C(4)). MS (FAB): 838 ($[M+K]^+$, 2.0%); 800 ($[M+H]^+$, 16.6%); 799 ($[M]^+$, 31.1%); 303 ($[C-Ph(Ph-OCH_3)_2]^+$, 100%).

**2-*N*-((di-*n*-Butylamino)-methylene)-2′/3′-O-*tert*-butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-l-guanosine (4c/c′).** **3c** (480 mg, 0.66 mmol) was treated as described as above. Purification by chromatography (toluene/EtOAc gradient from 50:50 to 20:80) afforded **4c** (194 mg, 0.23 mmol, 35%) and **4c**′ (188 mg, 0.22 mmol, 34%) as a yellowish foam. $R_f$ ($CHCl_3$/EtOAc 6:4): 0.25 (**4c**), 0.12 (**4c**′). $^1$H NMR ($CDCl_3$) (**4c**): −0.13 (s, 3H, $CH_3$); 0.03 (s, 3H, $CH_3$); 0.85 (s, 9H, *t*Bu); 0.94–0.99 (m, 6H, $CH_3CH_2$); 1.29–1.39 (m, 4H, $CH_3CH_2$); 1.55–1.68 (m, 4H, $CH_3CH_2CH_3CH_2$); 2.75 (d, 1H, $^3J$=3.9 Hz, HOC(3′)); 3.26–3.50 (m, 6H, $CH_2N$, $H_2C(5')$); 3.79 (s, 6H, $CH_3O$); 4.19–4.25 (m, 1H, HC(4′)); 4.28–4.32 (m, 1H, HC(3′)); 4.66 (t, 1H, $^3J$=5.4 Hz, HC(2′)); 6.00 (d, 1H, $^3J$=5.7 Hz, HC(1′)); 6.80–7.44 (m, 13H, Ph); 7.80 (s, 1H, HC(8)); 8.49 (br s, 1H, NH); 8.57 (s, 1H, NCH). $^{13}$C NMR ($CDCl_3$) (**4c**): −5.17, −4.99 (($CH_3)_2$Si); 13.67, 13.77 (*C*$H_3CH_2$); 17.88 (*C*($CH_3)_3$); 19.73, 20.11 ($CH_3CH_2$); 25.56 ((*CH*$_3)_3$C); 28.95, 30.98 ($CH_3CH_2CH_2$); 45.64, 52.20 ($CH_2N$); 55.24 ($CH_3O$); 63.64 (C(5′)); 71.72 (C(3′); 77.20 (C(2′)); 83.78 (C(4′)); 86.69 (C(1′)); 86.74 (*C*-Ph($PhOCH_3)_2$); 120.42

(C(5)); 113.27, 127.01–135.74, 157.62 (Ph); 144.42 (C(8)); 150.55 (C(4)); 156.84, 157.54 (C(6) or C(2)); 158.60 (NCH). $^1$H NMR ($CDCl_3$) (**4c**′): −0.02 (s, 3H, $CH_3$); 0.07 (s, 3H, $CH_3$); 0.87 (s, 9H, *t*Bu); 0.92–0.98 (m, 6H, $CH_3CH_2$); 1.27–1.39 (m, 4H, $CH_3CH_2$); 1.55–1.66 (m, 4H, $CH_3CH_2CH_2$); 3.22–3.50 (2m, 6H, $CH_2N$, $H_2C(5')$); 3.78 (s, 6H, $CH_3O$); 4.14 (m, 1H, HC(4′)); 4.36–4.42 (m, 2H, HC(2′), HC(3′)); 6.01 (d, 1H, $^3J$=4.5 Hz, HC(1′)); 6.80–7.43 (m, 13H, Ph); 7.53 (s, 1H, HC(8)); 8.58 (s, 1H, NCH); 8.74 (br s, 1H, NH). $^{13}$C NMR ($CDCl_3$) (**4c**′): −4.85, −4.77 ($(CH_3)_2Si$); 13.60, 13.74 ($CH_3CH_2$); 17.98 ($C(CH_3)_3$); 19.68, 20.10 ($CH_3CH_2$); 25.68 ($(CH_3)_3C$); 28.90, 30.85 ($CH_3CH_2CH_2$); 45.69, 52.18 ($CH_2N$); 55.19 ($CH_3O$); 62.84 (C(5′)); 71.93 (C(3′); 75.18 (C(2′)); 83.89 (C(4′)); 86.62 (C−Ph($PhOCH_3$)$_2$); 87.70 (C(1′)); 120.54 (C(5)); 113.21, 126.95–135.73,157.64 (Ph); 144.24 (C(8)); 150.33 (C(4)); 156.94, 157.60 (C(6) or C(2)); 158.57 (NCH). MS (FAB): 877 ($[M+K]^+$, 5.6%); 839 ($[M+H]^+$, 8.7%); 303 ($[C-Ph(Ph-OCH_3)_2]^+$, 100%).

**2′-O-*tert*-Butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-l-uridine 3′-O-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (5a).** Compound **4a** (77 mg, 0.12 mmol) was dried overnight under high vacuum. THF (0.5 ml, freshly distilled) was added under Ar followed by 2,4,6-collidine (116 μl, 0.87 mmol) and *N*-methylimidazole (5 μl, 0.06 mmol). 2-Cyanoethyl diisopropylphosphoramidochloridite (65 μl, 0.29 mmol) was added dropwise over 5 min. After stirring for 1 h at room temperature, the mixture was diluted with EtOAc (35 ml), then extracted by $NaHCO_3$ solution and brine. After drying over $Na_2SO_4$ and evaporation, purification by chromatography (EtOAc/hex/TEA 50:50:1) afforded **5a** (87 mg, 0.10 mmol, 88%) as a white powder after solubilisation in benzene, filtration through a Millipore filter (0.45 μm) and lyophilisation. $R_f$ (EtOAc/hex 1:1): 0.47, 0.42. Mixture of diastereoisomers: $^1$H NMR ($CDCl_3$): 0.08–0.15 (4s, 6H, $Si(CH_3)_2$); 0.93, 0.92 (2s, 9H, *t*Bu); 1.02–1.18 (2m, 12H, $(CH_3)_2CH$); 2.41–2.67 (2m, 2H, $CH_2CN$); 3.38–3.61 (2m, 4H, $H_2C(5')$, $(CH_3)_2CH$); 3.79–3.98 (m, 8H, $CH_3O$, $CH_2O$); 4.22–4.48 (3m, 3H, HC(4′), HC(2′), HC(3′)); 5.27–5.33 (2d, 1H, $^3J$=8.2 Hz, HC(5)); 5.90–5.99 (2d, 1H, $^3J$=4.8 Hz, HC(1′)); 6.83–7.43 (2m, 13H, Ph); 7.91–7.49 (2d, 1H, $^3J$=8.4 Hz, HC(6)); 8.04 (br s, 1H, NH). $^{31}$P NMR ($CDCl_3$): 149.83, 149.34.

**6-*N*-((di-*n*-Butylamino)-methylene)-2′-O-*tert*-butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-l-cytidine 3′-O-(2-cyanoethyl-*N,N*,-diisopropyl)phosphoramidite (5b).** Compound **4b** (117 mg, 0.15 mmol) was treated as described above. Purification by chromatography (EtOAc/hex/TEA 50:50:1) afforded **5b** (106 mg, 0.11 mmol, 73%) as a white powder after solubilisation in benzene, filtration through a Millipore filter (0.45 μm) and lyophilisation. $R_f$ (EtOAc/hex 1:1): 0.12, 0.22. Mixture of diastereoisomers: $^1$H NMR ($CDCl_3$): 0.01–0.26 (4s, 6H, $Si(CH_3)_2$); 0.89–1.18 (2m, 27H, *t*Bu, $(CH_3)_2CH$, $CH_3CH_2$); 1.26–1.38 (m, 4H, $CH_3CH_2$); 1.54–1.64 (m, 4H, $CH_3CH_2CH_2$); 2.35–2.63 (2m, 2H, $CH_2CN$); 3.31–4.41 (m, 19H, $CH_2N$, $H_2C(5')$, $(CH_3)_2CH$, $CH_3O$, $CH_2O$, HC(4′)), HC(3′), HC(2′)); 5.69–5.73 (m, 1H, HC(5)); 5.87–6.01 (2s, 1H, HC(1′)); 6.82–7.49 (m, 13H, Ph); 8.03–8.23 (2d, 1H, $^3J$=7.4 Hz, HC(6)); 8.86, 8.88 (2s, 1H, NCH). $^{31}$P NMR ($CDCl_3$): 149.31, 148.91.

**2-*N*-((di-*n*-Butylamino)-methylene)-2′-O-*tert*-butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-l-guanosine 3′-O-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (5c).** Compound **4c** (115 mg, 0.14 mmol) was treated as described above. Purification by chromatography (EtOAc/TEA 1%) afforded **5c** (128 mg, 0.13 mmol, 91%) as a white powder after solubilization in benzene, filtration through a Millipore filter (0.45 μm) and lyophilization. $R_f$ (EtOAc): 0.36. Mixture of diastereoisomers: $^1$H NMR ($CDCl_3$): −0.14–0.07 (4s, 6H, $Si(CH_3)_2$); 0.82, 0.83 (2s, 9H, *t*Bu); 0.89–1.O3 (m, 18H, $(CH_3)_2CH$, $CH_3CH_2$); 1.15–1.42 (m, 4H, $CH_3CH_2$); 1.53–1.65 (m, 4H, $CH_3CH_2CH_2$); 2.18–2.61 (2m, 2H, $CH_2CN$); 3.20–3.75 (2m, 8H, $CH_2N$, $H_2C(5')$, $(CH_3)_2CH$); 3.78–4.02 (m, 8H, $CH_3O$, $CH_2O$); 4.29–4.66 (3m, 3H, HC(4′), HC(3′), HC(2′)); 5.99–6.06 (2d, 1H, $^3J$=6.1 Hz, HC(1′)); 6.80–7.47 (m, 13H, Ph); 7.84, 7.89 (2s, 1H, HC(8)); 8.42 (br s, 1H, NH); 8.57, 8.59 (2s, 1H, NCH). $^{31}$P NMR ($CDCl_3$): 189.88, 188.51.

## Synthesis of the anchoring l-thymidine derivative

**2′,3′,5′-tri-O-Benzoyl-5-methyl-l-uridine (7).** 1-O-Acetyl-2,3,5-tri-O-benzoyl-β-l-ribofuranose (**6**) (1.50 g, 2.97 mmol) and thymine (1.13 g, 8.92 mmol) were coevaporated three times with anhydrous $CH_3CN$ under Ar. Absolute $CH_3CN$ (40 ml) and BSA (4.36 ml, 17.84 mmol) were added at room temperature. The mixture was stirred at 65°C until the base was dissolved. TMSOTf (0.81 ml, 4.46 mmol) was added and the mixture was stirred overnight under Ar at 65°C. $CH_2Cl_2$ (150 ml) was added and washed with sat. $NaHCO_3$ solution. After drying over $Na_2SO_4$ and evaporation, purification by chromatography (PE/EtOAc 5:5) afforded **7** (1.66 g, 2.91 mmol, 98%) as a white foam. $R_f$ (PE/EtOAc 5:5): 0.55. $^1$H NMR ($CDCl_3$): 1.60 (s, 3H, $CH_3$); 4.65 (dd, 1H, $^2J_{5'a,5'b}$=11.8 Hz, $^3J_{5'a,4'}$=3.5 Hz, HaC(5′)); 4.67–4.71 (m, 1H, HC(4′)); 4.88 (dd, 1H, $^2J_{5'b,5'a}$=11.8 Hz, $^3J_{5'b,4'}$=2.3 Hz, HbC(5′)); 5.76 (t, 1H, $^3J$=6.3, 3.1 Hz, HC(2′)); 5.92 (dd, 1H, $^3J$=6.0, 3.7 Hz, HC(3′)); 6.43 (d, 1H, $^3J$=6.3 Hz, HC(1′)); 7.16 (s, 1H, HC(6)); 7.33–8.15 (m, 15H, Ph); 8.95 (s, 1H, NH). $^{13}$C NMR ($CDCl_3$): 12.07 ($CH_3$); 63.92 (C(5′)); 71.42 (C(3′)); 73.35 (C(2′)); 80.59 (C(4′)); 86.91 (C(1′)); 112.18 (C(5)); 128.34–133.76 (Ph). 134.81 (C(6)); 150.31 (C(2)); 163.31 (C(4)); 165.31, 165.39, 165.97 (Ph*C*O). MS (FAB): 609 ($[M+K]^+$, 4.0%); 571 ($[M+H]^+$, 4.4%); 445 ($[M-base]^+$, 10.4%); 105 ($[PhCO]^+$; 100%).

**5-Methyl-l-uridine (8).** Compound **7** (1.64 g, 2.87 mmol) was dissolved in 0.1 M NaOMe/MeOH (60 ml). The mixture was stirred overnight at room temperature, then neutralised with a minimal amount of Dowex resin ($H^+$ form). After filtration and evaporation, water (40 ml) was added and extracted with $CH_2Cl_2$ (10×2 ml). The water layer was evaporated to give **8** (720 mg, 2.79 mmol, 97%) as a pale yellow powder after lyophilisation. Rf (EtOAc/MeOH/$H_2O$ 8:1:0.1): 0.34. Mp: 180–182°C. $^1$H NMR ($CD_3OD$): 1.87 (s, 3H, $CH_3$); 3.74 (dd, 1H, $^2J_{5a',5'b}$=12.3 Hz, $^3J_{5'a,4'}$=3.1 Hz, HaC(5′)); 3.85 (dd, 1H, $^2J_{5'a,5'b}$=12.3 Hz, $^3J_{5'b,4'}$=2.7 Hz, HbC(5′)); 3.97–4.01 (m, 1H, HC(4′)); 4.14–4.20 (m, 2H, HC(2′), HC(3′)); 5.89 (d, 1H, $^3J$=4.5 Hz, HC(1′)); 7.84 (s, 1H, HC(6)). $^{13}$C NMR ($CD_3OD$): 12.39 ($CH_3$); 62.32 (C(5′)); 71.30 (C(3′)); 75.54 (C(2′)); 86.32 (C(4′)); 90.45 (C(1′)); 111.46 (C(5));

138.43 (C(6)); 152.66 (C(2)); 166.40 (C(4)). MS (FAB): 297 ([M+K]$^+$, 35.4%); 259 ([M+H]$^+$, 31.8%); 133 ([M−base]$^+$, 13.9%); 43 (100%).

**3′,5′-O-(1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl)-5-methyl-l-uridine (9).** Compound **8** (722 mg, 2.79 mmol) was coevaporated three times with anhydrous pyr under Ar and dissolved in pyr (40 ml). DMAP (341 mg, 2.79 mmol) was added and the mixture was stirred completely dissolved. 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (0.92 ml, 2.94 mmol) was added dropwise via syringe. The mixture was stirred overnight at room temperature under Ar. The solvents were evaporated, then $NaHCO_3$-solution was added and extracted with $CH_2Cl_2$ (150 ml). After drying over $Na_2SO_4$ and evaporation, purification by chromatography (PE/EtOAc 5:5) gave **9** (1.15 g, 2.31 mmol, 82%) as a white foam. $R_f$ (PE/EtOAc 5:5): 0.56. $^1$H NMR ($CDCl_3$): 1.03–1.10 (m, 28H, $(CH_3)_2CH$); 1.91 (s, 3H, $CH_3$); 3.25 (s, 1H, HO−C(2′)); 3.98–4.04 (d"d", 1H, $^2J$=13.0 Hz, HaC(5′)); 4.08 (dt 1H, $^3J$=8.5, 2.8 Hz, HC(4′)); 4.16–4.22 (m, 2H, HbC(5′), HC(2′)); 4.41 (dd, 1H, $^3J$=8.5, 5.2 Hz, HC(3′)); 5.71 (s, 1H, HC(1′)); 7.40 (s, 1H, HC(6)); 8.23 (s, 1H, NH). $^{13}$C NMR ($CDCl_3$): 12.56, 12.73, 12.99, 13.44 ($CH_3$, $(CH_3)_2CH$); 16.87, 16.97, 17.01, 17.08, 17.25, 17.37, 17.43 ($(CH_3)_2CH$); 60.52 (C(5′)); 69.29 (C(3′)); 75.02 (C(2′)); 81.95 (C(4′)); 91.15 (C(1′)); 110.57 (C(5)); 135.82 (C(6)); 149.98 (C(2)); 163.72 (C(4)). MS (FAB): 539 ([M+K]$^+$, 64.0%); 501 ([M+H]$^+$, 100%); 375 ([M−base]$^+$, 15.2%).

**3′,5′-O-(1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl)-5-methyl-2′-O-(phenoxythiocarbonyl)-l-uridine (10).** Compound **9** (1.14 g, 2.27 mmol) was coevaporated two times with anhydrous pyr under Ar, then dissolved in abs. $CH_3CN$ (80 ml). DMAP (2.78 g, 22.73 mmol) was added at 0°C (ice bath). The mixture was stirred 15 min at 0°C then phenylcarbonochloridathioate (0.46 ml, 3.41 mmol) was added dropwise. The mixture was stirred overnight at room temperature then evaporated to dryness. EtOAc was added and extracted with water and $NaHCO_3$-solution. After drying over $Na_2SO_4$ and evaporation, purification by chromatography (PE/EtOAc 8:2 and 5:5) afforded **10** (1.21 g, 2.00 mmol, 88%) as a yellowish oil. Rf (PE/EtOAc 5:5): 0.81. $^1$H NMR ($CDCl_3$): 1.04–1.11 (m, 28H, $(CH_3)_2CH$); 1.93 (s, 3H, $CH_3$); 4.01–4.26 (m, 3H, HC(4′), $H_2C(5')$); 4.61 (dd, 1H, $^3J$=9.2, 5.0 Hz, HC(3′)); 5.91 (s, 1H, HC(1′)); 6.01 (d, 1H, $^3J$=5.1 Hz, HC(2′)); 7.10–7.44 (m, 6H, Ph, HC(6)); 8.27 (s, 1H, NH). $^{13}$C NMR ($CDCl_3$): 12.58, 12.74, 12.86, 12.95, 13.43 ($CH(CH_3)_2$, $CH_3$); 16.92, 16.96, 17.03, 17.26, 17.37, 17.41 ($(CH_3)_2CH$); 59.54 (C(5′)); 68.42 (C(3′)); 82.09 (C(4′)); 83.84 (C(2′)); 88.77 (C(1′)); 111.05 (C(5)); 121.77, 126.69, 129.56, 153.45 (Ph); 135.29 (C(6)); 149.53 (C(2)); 163.30 (C(4)); 193.85 (C(S)). MS (FAB): 511 ([M−OPh]$^+$, 28.2%); 483 (100%).

**3′,5′-O-(1,1,3,3-Tetraisopropyldisiloxan-1,3-diyl)-l-thymidine (11).** Compound **10** (906 mg, 1.50 mmol) was coevaporated three times with anhydrous toluene under Ar, dissolved in toluene (35 ml) and degassed with Ar for 30 min. In a second flask, AIBN (123 mg, 0.75 mmol) and $Bu_3SnH$ (1.07 ml, 4.49 mmol) in absolute toluene (7 ml) were degassed with Ar for 30 min. The first flask was then heated to 80°C, the second solution was added dropwise via syringe. The mixture was heated to 90°C for 4 h. After cooling to room temperature, the mixture was evaporated, the residue dissolved in $CH_2Cl_2$ and extracted with $NaHCO_3$-solution. After drying over $Na_2SO_4$ and evaporation, purification by chromatography (PE/EtOAc 9:1 and 6:4) afforded **11** (600 mg, 1.23 mmol, 82%) as a white foam. $R_f$ (PE/EtOAc 5:5): 0.66. $^1$H NMR ($CDCl_3$): 1.02–1.13 (m, 28H, $(CH_3)_2CH$); 1.92 (s, 1H, $CH_3$); 2.55 (m, 1H, $H_AC(2')$); 2.49 (m, 1H, $H_BC(2')$); 3.75 (dt, 1H, $^3J$=2.8, 8.0 Hz, HC(3′)); 4.03 (dd, 1H, $^2J_{5'a,5'b}$=13.1 Hz, $^3J_{5'a,4'}$=3.0 Hz, HaC(5′)); 4.11 (dd, 1H, $^2J_{5'b,5'a}$=13.1 Hz, $^3J_{5'b,4'}$=2.5 Hz, HbC(5′)); 4.49 (dt, 1H, $^3J$=9.1, 7.8 Hz, HC(4′)); 6.08 (dd, 1H, $^3J$=6.2, 2.8 Hz, HC(1′)); 7.42 (s, 1H, HC(6)); 8.56 (s, 1H, NH). $^{13}$C NMR ($CDCl_3$): 12.46, 12.77, 13.01, 13.48 ($CH(CH_3)_2$, $CH_3$); 16.76, 16.84, 16.97, 17.09, 17.27, 17.33, 17.41, 17.45 ($(CH_3)_2CH$); 39.78 (C(2′)); 60.57 (C(5′)); 67.57 (C(3′)); 83.79 (C(4′)); 84.96 (C(1′)); 110.51 (C(5)), 135.21 (C(6)); 149.98 (C(2)); 163.62 (C(4)). MS (FAB): 488 ([M]$^+$, 0.3%); 81 (100%).

**l-Thymidine (12).** Compound **11** (584 mg, 1.20 mmol) was dissolved in THF (30 ml), then $Bu_4NF$ (1 M in THF) (2.63 ml, 2.63 mmol) was added. The mixture was stirred 2 h at room temperature, then evaporated to dryness. Purification by chromatography (EtOAc/MeOH gradient from 100:0 to 90:5) gave **12** (217 mg, 0.90 mmol, 75%) as a white powder. $R_f$ (EtOAc/MeOH 9:1): 0.43; mp: 179–181°C. $[\alpha]_D^{25}=-26.3\pm0.5$ ($c$=0.37, MeOH). $^1$H NMR ($CD_3OD$): 1.88 (s, 3H, $CH_3$); 2.19–2.26 (m, 2H, $H_AC(2')$); 3.73 (dd, 1H, $^2J_{5'a,5'b}$=11.9 Hz, $^3J_{5'a,4'}$=3.7 Hz, HaC(5′)); 3.90 (dd, 1H, $^2J_{5'b,5'a}$=12.1 Hz, $^3J_{5'b,4'}$=3.1 Hz, HbC(5′)); 3.90 (dd, 1H, $^3J$=6.7, 3.3 Hz, HC(4′)); 4.39 (dt, 1H, $^3J$=5.7, 3.5 Hz, HC(3′)); 6.28 (t, 1H, $^3J$=6.8 Hz, HC(1′)); 7.81 (s, 1H, HC(6)). $^{13}$C NMR ($CD_3OD$): 12.44 ($CH_3$); 41.22 (C(2′)); 62.84 (C(5′)); 72.21 (C(3′)); 86.25 (C(1′)); 88.82 (C(4′)); 111.51 (C(5)); 138.16 (C(6)); 152.37 (C(2)); 166.42 (C(4)). MS (FAB): 243 ([M+H]$^+$, 19.3%); 242 ([M]$^+$, 100%); 117 ([M−base]$^+$, 2.1%).

**5′-O-(4,4′-Dimethoxytrityl)-l-thymidine (13).** Compound **12** (149 mg, 0.62 mmol) was coevaporated three times with anhydrous pyr, dissolved in pyr (15 ml), then DMAP (19 mg, 0.15 mmol), DMTCl (coevaporated with anhydrous pyr, 250 mg, 0.74 mmol, dissolved in pyr (3 ml)) were added. The mixture was stirred for 4 h at room temperature, then MeOH (1 ml) was added and the mixture was evaporated. The residue was dissolved in EtOAc and washed with sat. $NaHCO_3$-solution. After drying over $Na_2SO_4$ and evaporation, purification by chromatography (EtOAc, 0.1% TEA) gave **13** (271 mg, 0.50 mmol, 81%) as a light-yellow foam. $R_f$ (EtOAc): 0.58. $^1$H NMR ($CDCl_3$): 1.47 (s, 3H, $CH_3$); 2.61–2.35 (m, 1H, $H_AC(2')$); 2.39–2;46 (m, 1H, $H_BC(2')$); 3.37 (dd, 1H, $^2J_{5'a,5'b}$=10.5 Hz, $^3J_{5'a,4'}$=3.0 Hz, HaC(5′)); 3.47 (dd, 1H, $^2J_{5'b,5'a}$=10.6 Hz, $^3J_{5'b,4'}$=3.2 Hz, HbC(5′)); 3.78 (s, 6H, $OCH_3$); 4.05–4.08 (m, 1H, HC(4′)); 4.57 (ddd, 1H, $^3J$=6.1, 3.0, 2.9 Hz, HC(3′)); 6.42 (t, 1H, $^3J$=5.8 Hz, HC(1′)); 6.82–7.41 (m, 13H, Ph); 7.59 (s, 1H, HC(6)). $^{13}$C NMR ($CDCl_3$): 11.84 ($CH_3$); 44.99 (C(2′)); 55.24 ($OCH_3$); 63.52 (C(5′)); 72.53 (C(3′)); 84.78 (C(4′)); 86.25 ($C$–Ph($PhOCH_3$)$_2$); 86.99 (C(1′)); 111.29 (C(5)); 113.31–135.69, 158.76 (Ph); 144.36 (C(6)); 150.47 (C(2)); 163.81 (C(4)). MS (FAB): 583 ([M+K]$^+$, 6.2%); 545 ([M+H]$^+$, 1.4%); 271 ([M−C(Ph($PhOCH_3$)$_2$]$^+$, 1.4%); 303 (100%).

**5′-O-(4,4′-Dimethoxytrityl)-l-thymidine 3′-O-succinate (14).** To a solution of dried **13** (270 mg, 0.50 mmol) in 1,2-dichloroethane (1 ml), DMAP (30 mg, 0.25 mmol), succinic anhydride (88 mg, 0.74 mmol) and TEA (69 μl, 0.50 mmol) were added. The reaction mixture was stirred at 50°C for 20 min and cooled, showing the formation of a single, more polar product on tlc. The solution was further diluted with $CH_2Cl_2$ (15 ml), washed with ice-cold aqueous solution of 10% citric acid (10×3 ml) and water (10×2 ml). After drying over $Na_2SO_4$ and evaporation, purification by chromatography ($CH_2Cl_2$/MeOH gradient from 98:2 to 92:8) furnished **14** (279 mg, 0.43 mmol, 87%) as a white foam. $R_f$ ($CH_2Cl_2$/MeOH 9:1): 0.58. $^1$H NMR ($CDCl_3$): 1.38 (s, 3H, $CH_3$); 2.37–2.59 (2 m, 2H, $H_2C(2')$); 2.60–2.66 (m, 4H, $(CH_2)_2$); 3.41 (dd, 1H, $^2J_{5'a,5'b}$=10.5 Hz, $^3J_{5'a,4'}$=2.4 Hz, HaC(5′)); 3.48 (dd, 1H, $^2J_{5'b,5'a}$=10.6 Hz, $^3J_{5'b,4'}$=2.0 Hz, HbC(5′)); 3.78 (s, 6H, $OCH_3$); 4.17–4.18 (m, 1H, HC(4′)); 5.44 (d, 1H, $^3J$=5.3 Hz, HC(3′)); 6.42 (dd, 1H, $^3J$=9.0, 5.4 Hz, HC(1′)); 6.81–7.40 (m, 13H, Ph); 7.61 (s, 1H, HC(6)). $^{13}$C NMR ($CDCl_3$): 11.55 ($CH_3$); 30.83, 31.74 $(CH_2)_2$; 37.94 (C(2′)); 55.22 ($OCH_3$); 63.79 (C(5′)); 75.09 (C(3′)); 84.02 (C(4′)); 84.40 (1′); 87.08 (*C*–Ph($PhOCH_3$)$_2$); 111.47 (C(5)); 113.28–135.53, 158.72 (Ph); 144.26 (C(6)); 150.35 (C(2)); 163.61 (C(4)); 173.35, 177.85 (CO). MS (FAB): 683 ($[M+K]^+$, 2.7%); 645 ($[M+H]^+$, 1.3%); 303 (100%).

**5′-O-(4,4′-Dimethoxytrityl)-l-thymidine 3′-O-(succinyl-amino-*N*-hexamethylene-*N*′-aminomethyl)polystyrene (15).** Compound **14** (64 mg, 0.1 mmol) and DMAP (12.3 mg, 0.1 mmol) were weighed into a septum-sealed vial fitted with a small needle and kept over night under continuous vacuum. The vacuum was released with dry Ar, the needle removed, and anhydrous dichloromethane (2.5 ml) was added followed by HMDIC (16 μl, 0.1 mmol). The reaction was allowed to proceed at room temperature. After 15 min, the vial was opened under Ar and the polymer support (500 mg, 26.8 μmol/g) and *N*-ethyl diisopropylamine (17 μl, 0.1 mmol) were added. The vial was closed with a Teflon septum and gently shaken during 48 h. The polymer support was then filtered, washed with dichloromethane (10×3 ml) and $Et_2O$ (10×3 ml). The polymer support was suspended in pyr/water (25 ml, 4:1 v/v) for 10 min at room temperature, washed with pyr (10×3 ml), and suspended in $Ac_2O$/TEA/*N*-methylimidazole/$CH_2Cl_2$ (16 ml, 1:1:0.3:6) for 30 min at room temperature. After filtration, the polymer support was washed with dichloromethane (10×2 ml) and $Et_2O$ (10×2 ml) and dried over night under high vacuum. Loading: 5.00 mg **15** were suspended in 3% TCA/$CH_2Cl_2$ (5 ml). After 5 min, the absorbance of the supernatant was measured at 504 nm (1 cm path length). $A_{504}$=0.996, $\epsilon_{504}$ (trityl$^+$)=72′678 $M^{-1}$ $cm^{-1}$=>13.7 μmol trityl/g resin.

## Synthesis of d- and l-oligonucleotides

The synthesis of the oligonucleotides was carried out on an Applied Biosystems Inc. (ABI) DNA/RNA-Synthesizer, Model 392. Solid support: aminomethylpolystyrene (product no. 360865C, Lot: 9609225GB, capacity of loading: 26.8 μmol/g as measured by a ninhydrin test according to Kaiser). d-Monomers (TOM™, Xeragon, Zürich, Switzerland). Coupling reagent (Xeragon): 0.25 M 5-Benzylthio-1*H*-tetrazole/$CH_3CN$; other reagents (ABI): 3% TCA/$CH_2Cl_2$ (detritylation), $Ac_2O$/lutidine/THF (Cap A), *N*-methylimidazole/THF (Cap B), 0.02 M $I_2$/$H_2O$/pyr/THF (oxidation), 40% aq. $CH_3NH_2$ (Fluka) for the deprotection of the bases and the cleavage from the solid support.

Some modifications to the standard protocol were applied in our oligonucleotide synthesis. For the d-nonamer: 77 mg (1.3 μmol) **15** (D), use of 5-benzylthiotetrazole instead of tetrazole in the coupling step, coupling time: 300 s, capping step before and after the oxidation step, trityl off. For the l-nonamer: 54 mg (0.75 μmol) **15** (L), use of 5-benzylthiotetrazole instead of tetrazole in the coupling step, 10 equivalents of l-monomers (0.03 M), coupling time: 600 s, capping step before and after the oxidation step, trityl off.

The oligonucleotide synthesised was deprotected with approximately 1.5 ml of 40% aq. $CH_3NH_2$ solution during 2 h at room temperature on the DNA/RNA Synthesizer (modified END RNA-Procedure from ABI), then the solution was transferred into two Eppendorf tubes. The solution was evaporated on a SpeedVac for approx. 3 h. The residue (oil) was dissolved in 270 μl $NEt_3$·3HF and 90 μl DMF, shortly vortexed and left at 55°C in a thermomixer for 1.5 h. To quench the reaction, 30 μl $H_2O$ were added and the resulting solution was distributed in three Eppendorf tubes (130 μl each) and mixed with 1 ml of *n*-butanol each. The mixtures were shortly vortexed and left for at least 30 min at −20°C. The fine precipitates were centrifuged during 10 min at 14 000 U/min and at room temperature. The supernatant was removed and the combined pellets were dissolved in a total of 600 μl of buffer A (20 mM Na/K-phosphate buffer, pH 7.0, CIBA). $OD_{260}$ values of the crude oligonucleotides: 35/0.6 ml (d-nonamer), 42/0.6 ml (l-nonamer).

## HPLC purification: strong anion exchange (SAX)

Column: 125×8 mm Nucleogen® DEAE 500-7 (Macherey–Nagel). Eluent mixture: A: 20 mM Na/K-phosphate buffer, pH 7.0; B: 0.6 M NaCl in buffer A. Detection: 260 nm (analytical); 290 nm (preparative). Flow rate: 2 ml/min. Gradient: 0–5 min 0–10% B, 5–8 min 10–25% B, 8–20 min 25–30% B. The main peak eluted between 25 and 30% B. Retention time: ~19.5 min.

## Desalting

Column: 250×8 mm Eurospher® 100/5 $RP_{18}$ (Macherey–Nagel). Eluent: A: $H_2O$ (nanopure), B: $CH_3CN$/$H_2O$ (nanopure) 3:7; injector loop volume: 2 ml; flow rate: 3 ml/min; detection: 260 nm.

For complete desalting, an anion exchange fraction was loaded in approximately six 1.5-ml portions onto the column (2 ml/min A). After all RNA had been adsorbed, the excess salts were washed off the column with water (up to 6 ml/min). RNA counterions ($Na^+$/$K^+$) were exchanged by injecting 1 ml of 0.25 M $NH_4OAc$ (flow rate 2 ml/min), excess ammonium salt was washed off with water (5 ml/min) and desalted RNA ($NH_4^+$ form) was eluted by a step gradient to 100% B (3 ml/min). The collected RNA fraction was distributed over three Eppendorf tubes and evaporated on a SpeedVac until a volume of approximately 100 μl each

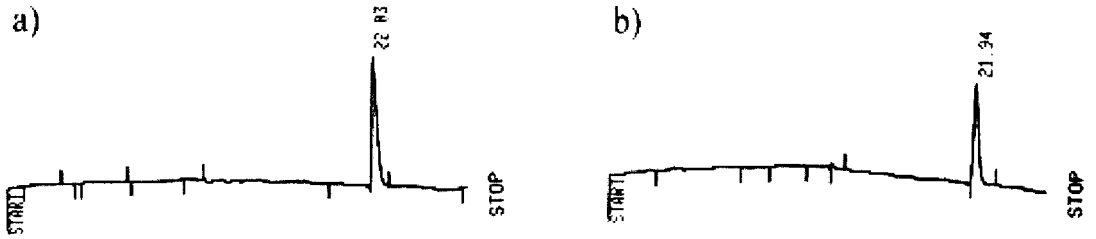



**Figure 4.** RP-HPLC: (a) d-nonamer; (b) l-nonamer.

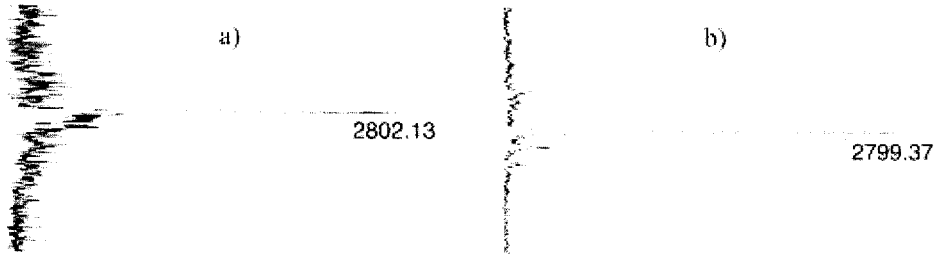



**Figure 5.** MALDI ToF MS. Theoretical mass of the oligoacid+$H^+$: 2805 Da (precision ±10 Da): (a) d-nonamer; (b) l-nonamer.

was reached (do not evaporate to dryness!). The tubes were combined into one Eppendorf tube and the stock solution was stored in the deep freezer (−20°C). $OD_{260}$ values of the stock solutions: 4/0.3 ml (d-nonamer); 6/0.4 ml (l-nonamer).

### Analysis: reversed-phase HPLC and MALDI ToF MS

Column: 250 mm×8 mm Eurospher® 100/5 (Macherey–Nagel). Eluent mixture: A: 0.1 M $NH_4OAc$, B: 30% $CH_3CN$ in buffer A. Detection: 260 nm. Gradient: 0–20 min: 0–20% B, 20–25 min: 20% B. The RP-Chromatograms are depicted in Fig. 4a (d-nonamer) and Fig. 4b (l-nonamer), the MALDI ToF mass spectra in Fig. 5.

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