Tuning Single Nucleotide Discrimination in Polymerase Chain Reactions (PCRs): Synthesis of Primer Probes Bearing Polar 4′-*C*-Modifications and Their Application in Allele-Specific PCR

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Abstract: There are many methods available for the detection of nucleotide variations in genetic material. Most of these methods are applied after amplification of the target genome sequence by the polymerase chain reaction (PCR). Many efforts are currently underway to develop techniques that can detect single nucleotide variations in genes either by means of, or without the need for, PCR. Allele-specific PCR (asPCR), which reports nucleotide variations based on either the presence or absence of a PCR-amplified DNA product, has the potential to combine target amplification and analysis in one single step. The principle of asPCR is based on the formation of matched or mismatched primer–target complexes by using allele-specific primer probes. PCR amplification by a DNA polymerase from matched 3′-primer termini proceeds, whereas a mismatch should obviate amplification. Given the recent advancements in real-time PCR, this technique should, in principle, allow single nucleotide variations to be detected online. However, this method is hampered by low selectivity, which necessitates tedious and costly manipulations. Recently, we reported that the selectivity of asPCR can be significantly increased through the employment of chemically modified primer probes. Here we report further significant advances in this area. We describe the synthesis of various primer probes that bear polar 4′-*C*-modified nucleotide residues at their 3′ termini, and their evaluation in real-time asPCR. We found that primer probes bearing a 4′-*C*-methoxymethylene modification have superior properties in the discrimination of single nucleotide variations by PCR.

Keywords: DNA replication · genomics · oligonucleotides · polymerase chain reactions · polymorphism

Introduction

The deciphering of the human genome sequence has facilitated studies of genome variation between individuals and the influence of these differences, such as single nucleotide polymorphisms (SNPs), on the predisposition to disease, and on the effects of drugs on different patients. So far, nearly 1.8 million SNPs have been discovered and characterized.[1] A specific knowledge of clinically relevant nucleotide variations may enable a particular therapy to be adapted to the respective genetic make-up of the patient, and should help to predict individual drug efficacies and/or toxicities.[2–15] Methods that permit the efficient and cost-effective diagnosis of relevant single nucleotide variations will further advance this field. Many methods for the detection of nucleotide variations have been described; however, because these exhibit a range of advantages and disadvantages, no general methodology has prevailed.[10–15] Most of these methods are applied after amplification of the target genome sequence by the polymerase chain reaction (PCR).[10–15]

Allele-specific PCR (asPCR), which reports nucleotide variations based on either the presence or absence of a DNA amplification product, has the potential to combine target amplification and analysis in one single step.[16–23] The principle of asPCR is based on the formation of matched or mismatched primer–target complexes through the use of allele-specific primer probes. PCR amplification catalyzed by a DNA polymerase proceeds from matched 3′-primer termini, whereas a primer–template mismatch should obviate amplification. However, there have been numerous reports indicating low selectivity of this approach, which necessitates the laborious and costly optimization of buffer conditions, as well as sequence and assay design.[20–23]

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Recently, we and others have reported that the selectivity of asPCR can be significantly increased by employing chemically modified primer probes.[24–28] By using primer probes that bear small, nonpolar 4′-*C*-modifications at their 3′ termini, together with a commercially available 3′-5′-exonuclease-deficient variant of a DNA polymerase from *Thermococcus litoralis* (Vent (exo−) DNA polymerase),[24–26] we observed a significantly higher amplification selectivity than was obtained with unmodified primer probes. We were able to show that detection of single nucleotide variations could be diagnosed by using real-time PCR,[29] in which fluorescent *SybrGreen I* detection provided a rapid and convenient tool to detect and analyze the degree of allele discrimination.

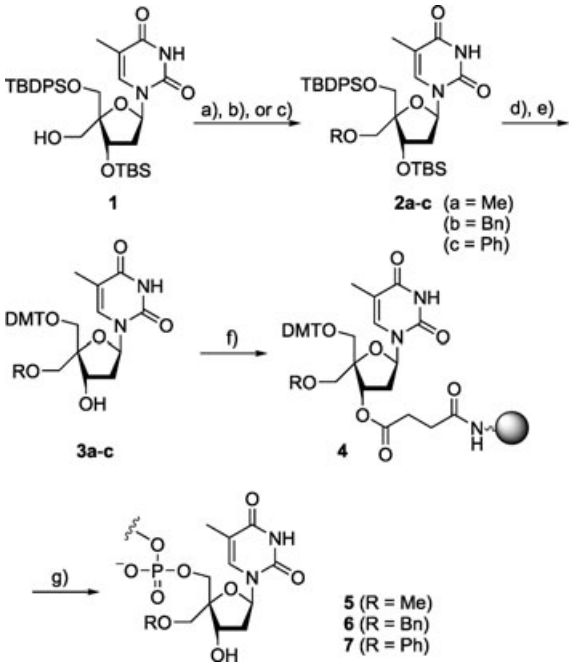
Here we describe the synthesis of various primer probes that bear polar 4′-*C*-modified nucleotide residues at their 3′ termini, and the evaluation of these probes by using real-time asPCR. We discovered that primer probes bearing a 4′-*C*-methoxymethylene modification have superior properties in the discrimination of single nucleotide variations by PCR.

Results and Discussion

Synthesis of 4′-*C*-modified primer probes: To explore the effects of polar 4′-*C*-substituents on the selectivity of single nucleotide discrimination in PCR we synthesized a variety of 4′-*C*-modified oligonucleotides. Our synthetic approach uses the known 4′-*C*-modified thymidine (**1**) as a versatile starting point for further diversification (Scheme 1). Compound **1** was chosen as it is readily available in gram quantities from a procedure published by Giese and co-workers.[30]

Compound **1** was converted into its methyl and benzyl ether (**2a**–**b**) by using the respective methyl or benzyl halogenides. The phenyl ether **2c** was synthesized by conversion of the alcohol **1** into the triflate[31,32] and subsequent treatment with sodium phenolate. Desilylation and 5′-*O*-tritylation of **2a**–**c** yielded **3a**–**c**, which were subsequently coupled to a succinylated long-chain alkyl amine-modified controlled pore glass (LCAA-CPG) support.[33] The solid supports **4a**–**c** were used in standard automated DNA synthesis to yield oligonucleotides **5**–**7** that carry the respective, modified thymidine moiety at their 3′ termini.[33] We also synthesized oligonucleotides bearing 4′-*C*-hydroxymethylene moieties at their 3′ termini by following a protocol published by Wengel and co-workers.[34] Attempts to synthesize the 4′-*C*-ethoxymethylene analogue from **1** failed; therefore, we developed the synthesis by the de novo construction of the nucleoside. This synthesis starts with the known ribose derivative **8** that was readily alkylated to form ethyl ether **9** (Scheme 2).[35,36]

Protection group manipulations involved cleavage of the acetyl group and conversion to the respective acetates.[35] The nucleobase was introduced by using a standard protocol for Vorbrüggen glycosylation to yield **10**.[37,38] Next, saponification and deoxygenation of the 2′-hydroxyl function was conducted to yield **11**. Deprotection gave **12**, which was

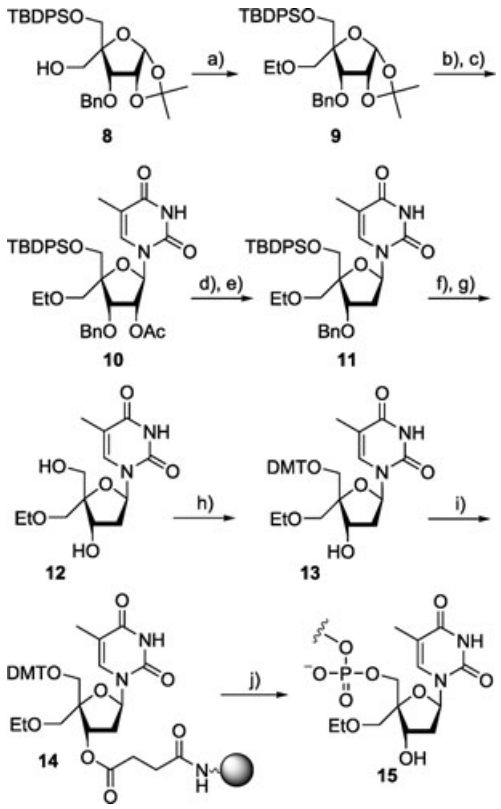


Scheme 1. a) NaH, MeI, THF (**2a**: 75%); b) NaH, BnBr/NaI, THF (**2b**: 40%); c) i. Tf_2O, pyridine, CH_2Cl_2, ii. PhOH, NaH, DMF (**2c**: 50%); d) 1 M TBAF, THF; e) DMTCl, DMAP, pyridine (**3a**: 54%, **3b**: 27%, **3c**: 55%); f) EDC, DMAP, succinylated LCAA-CPG, pyridine; then 4-nitrophenol; then piperidine; then acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B); g) i. oligonucleotide synthesis, ii. 33% NH_4OH. TBS = *tert*-butyldimethylsilyl; TBDPS = *tert*-butyldiphenylsilyl; DMT = 4,4′-dimethoxytrityl; DMAP = 4-(*N*,*N*-dimethylamino)-pyridine; LCAA-CPG = long-chain alkyl amine-modified controlled pore glass; EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; TBAF = tetra-*n*-butylammonium fluoride.

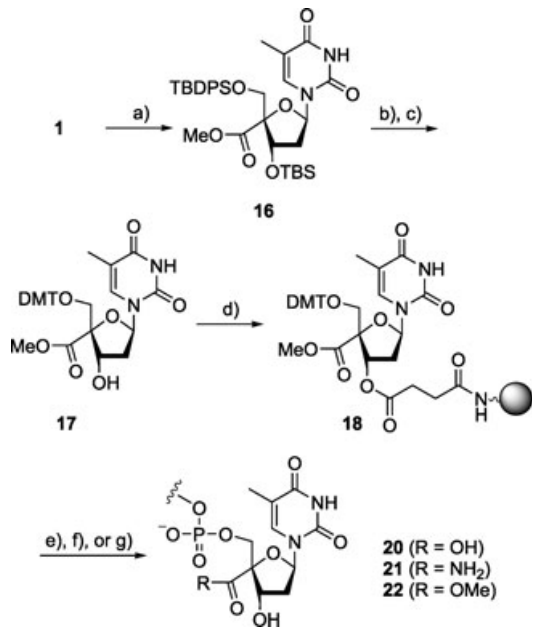
transformed into **13** by 5′-*O*-tritylation. Following coupling to a solid support, oligonucleotide synthesis was readily achieved by using **14** to yield **15**.

Next, we investigated the synthesis of various caboxylate-modified oligonucleotides from **1** (Scheme 3). Oxidation and ester formation yielded the methyl ester **16**.[39] Protection group manipulations produced the 5′-*O*-tritylated building block **17** that was subsequently coupled to a solid support to yield **18**. This was followed by automated DNA synthesis. To vary the carboxylate functionality, the resin-bound oligonucleotides were cleaved from the support and then deprotected under different conditions. For example, to obtain the carboxylate functionality in **20** the resin was treated with 0.5 M NaOH; for the synthesis of the amide **21**, standard deprotection with concentrated ammonia was employed; and to maintain the methyl ester functionality in **22**, 2 M NaOMe was used to cleave the oligonucleotides from the solid support, and also the protection groups. The integrities of the oligonucleotides were confirmed by high resolution mass spectrometric analysis (HRMS).

Single nucleotide discrimination by real-time PCR using unmodified versus 4′-*C*-modified primer probes: We conducted two reactions simultaneously and in parallel: One PCR tem-



Scheme 2. a) NaH, EtI, DMF, 63%; b) i. 80% AcOH, TFA, ii. Ac_2O, DMAP, pyridine; c) thymine, BSA, TMSOTf, ACN, 32% over all steps; d) NaOMe, MeOH; e) PhOCSCl, DMAP, ACN; f) AIBN, nBu$_3$SnH, toluene, 65% after both steps; g) i. 1 M TBAF, THF, ii. $Pd(OH)_2/C$, H_2, ethanol, 69% over both steps; h) DMTCl, DMAP, pyridine, 25%; i) EDC, DMAP, succinylated LCAA-CPG, pyridine; then 4-nitrophenol; then piperidine; then acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B); j) i. oligonucleotide synthesis, ii. 33% NH_4OH. BSA = *N,O*-bis(trimethylsilyl)acetamide; AIBN = 2,2′-azobisisobutyronitrile.



Scheme 3. a) i. PDC, powdered molecular sieve (4 Å), DMF, ii. EDC, DMAP, MeOH, CH_2Cl_2, 44% over both steps; b) 1 M TABF, THF; c) DMTCl, DMAP, pyridine, 56% over both steps; d) EDC, DMAP, succinylated LCAA-CPG, pyridine; then 4-nitrophenol; then piperidine; then acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B); e) i. oligonucleotide synthesis, ii. 0.5 M NaOH, then 2 M TEAA (pH 7) to yield **20**; f) i. oligonucleotide synthesis, ii. 33% NH_4OH to yield **21**; g) i. oligonucleotide synthesis, ii. 2 M NaOMe, MeOH, then 2 M TEAA (pH 7) to yield **22**. PDC = pyridinium dichromate; TEAA = triethylammonium acetate buffer.

plate contained a dA residue in the position opposite to the 3′-terminal thymidine in the primer probe.[40] The other experiment employed the same primer probe and template strand; however, the sequence of the template included a dA-to-dG mutation opposite to the 3′-terminal thymidine moiety. Both set-ups contained the same reverse primer. The progress of the PCR was analyzed in real-time by using appropriate thermocycler equipment to measure the fluorescence of *SybrGreen I* in response to double-stranded DNA binding. We compared the efficacy of amplification of the respective primer probe by using Vent (exo−) DNA polymerase. Two crucial parameters were identified: the threshold crossing point (C_t) as a measure of amplification efficiency, and the difference in threshold crossing points (ΔC_t) of canonical versus noncanonical primer–template amplification as an indication of single nucleotide discrimination. The results are shown in Table 1 and, in part, in Figure 1.

Clearly, amplification efficiency and the ability to discriminate between single nucleotide mismatches varies according to the modified residue employed. The use of thymidines

Table 1. ΔC_t values obtained by using unmodified or 4′-*C*-modified primer probes and DNA template Far A versus Far G.

5′–AGGT$^{\mathbf{R}}$ (Primer probe) 3′–TCC**N**ACTAA– (Template)[a]			
	R	C_t(**N** = A)	ΔC_t
Far 1	H	17	0
Far 2	CH_2OH	17	2.5
Far 3	CH_2OCH_3	19	9
Far 4	$CH_2OCH_2CH_3$	n.a.[b]	–
Far 5	CH_2OBn	n.a.[b]	–
Far 6	CH_2OPh	n.a.[b]	–
Far 7	CO_2H	n.a.[b]	–
Far 8	$C(O)NH_2$	22	7
Far 9	$C(O)OCH_3$	n.a.[b]	–

[a] **N** = A or G for Far A or Far G, respectively. [b] n.a.: no amplification after 40 cycles of PCR.

that bear bulky ethers, methyl ester, or carboxylate 4′-*C*-modifications resulted in no significant amplification after 40 PCR cycles. The smaller 4′-*C*-hydroxymethylene functional group failed to induce significant discrimination between match and mismatch; however, the 4′-*C*-methoxymethylene modification gave superior results in the discrimination of single nucleotide variations by PCR.

Next, we studied the properties of 4′-*C*-methoxymethylene-modified primer probes in several clinically relevant se-

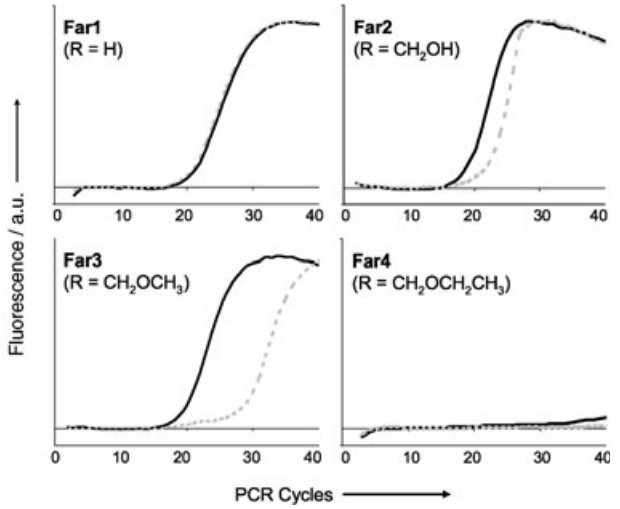


Figure 1. Results of real-time PCR experiments obtained by using primer probes bearing unmodified or 4′-*C*-modified thymidine residues at the 3′ terminus of the primer (R: 4′-*C*-modification as indicated in Table 1). PCR amplification in the presence of Far primer (as indicated), and target template Far A (solid line) or Far G (dashed line). All experiments were conducted under identical reaction conditions containing equal amounts of dNTPs, DNA substrates, and Vent (exo−) DNA polymerase.

quences,[41,42] and compared their properties with those of the recently identified, respective 4′-*C*-vinylated probes.[25] The results are depicted in Table 2.

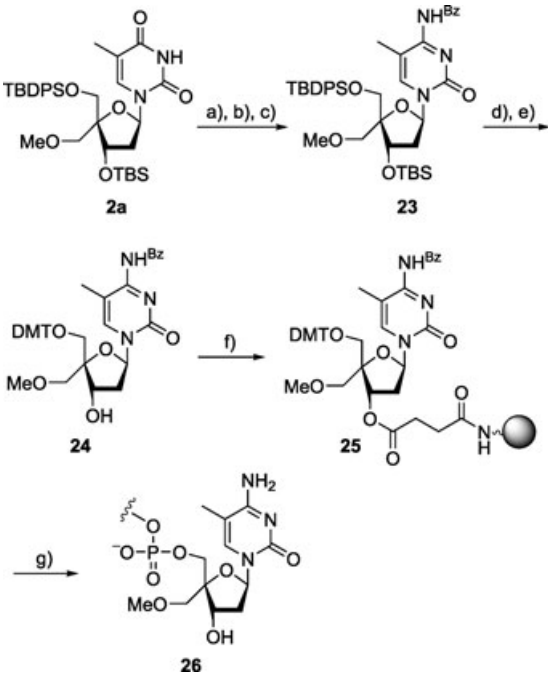
Table 2. ΔC_t values obtained by using unmodified or 4′-*C*-modified primer probes in various sequence contexts.

R	Far A vs Far G	Lei A vs Lei G	DPyD A vs DPyD G
H[a]	0	1	1.5
CH=CH$_2$[a]	8	10	4.5
CH$_2$OCH$_3$	9	12	14

[a] Results from ref. [25].

Interestingly, for all of the sequences investigated, the 4′-*C*-methoxymethylene-modified probes gave results superior to those of the known 4′-*C*-vinyl-modified probes.

Synthesis of 4′-*C*-methoxymethylene-5-methyl cytidine and its properties as primer probes: To perform genotyping experiments we synthesized the respective cytidine derivatives. Because 4′-*C*-methoxymethylene thymidine derivatives displayed the most discriminative properties (see above), we focused on the synthesis of the respective 2′-deoxycytidine derivatives. Our synthetic strategy was based on the conversion of uridine or thymidine derivatives into the respective cytidine analogues.[43–45] Thus, **2a** was converted into **23** by treatment with the 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl)-Et$_3$N-DMAP system, and subsequent aminolysis and benzoylation of the exocyclic amino function. Standard protection group manipulations yielded **24**, which was coupled to a solid support (**25**), from which oligonucleotides bearing 4′-*C*-methoxymethylene-5-methyl cytidine residues at the 3′ terminus (**26**) were readily available (Scheme 4).



Scheme 4. a) TPSCl, DMAP, Et$_3$N, ACN; b) 33% NH$_4$OH/ACN; c) Bz$_2$O, DMAP, pyridine, 63% over all steps; d) 1 M TBAF, THF; e) DMTCl, DMAP, pyridine, 71% over both steps; f) EDC, DMAP, succinylated LCAA-CPG, pyridine; then 4-nitrophenol; then piperidine; then acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B); g) i. oligonucleotide synthesis, ii. 33% NH$_4$OH. TPSCl = 2,4,6-triisopropylbenzenesulfonyl chloride.

We synthesized primer probes for the same sequence as that described above, and used real-time PCR to compare their efficiency in the discrimination of single nucleotide variations with those of an unmodified probe. We found that, as above, the use of modified probes led to a significant increase in selectivity in the detection of single nucleotide variants within PCR targets (Table 3).

Table 3. ΔC_t values obtained by using unmodified or 4′-*C*-modified primer probes containing **26** in various sequence contexts.

	Far G vs. Far A	Lei G vs. Lei A	DPyD G vs. DPyD A
dC	0	1	1
26	10	13	8

These results demonstrate that chemically modified primer probes are amplified in matched primer–template complexes significantly more efficiently than do their unmodified counterparts.

Conclusion

We have shown that primer probes bearing certain polar 4′-*C*-modifications can significantly increase single nucleotide discrimination by PCR. The 4′-*C*-methoxymethylene-modified nucleotides were shown to be ideally suited for this pur-

pose. These compounds are readily available and can be incorporated into DNA strands using standard oligonucleotide chemistry. The systems described supersede recently reported approaches and should be useful for the direct diagnosis by PCR of single nucleotide variations, such as single nucleotide polymorphisms or point mutations, without the need for further time-consuming and costly post-PCR analyses.

Experimental Section

General: All temperatures quoted are uncorrected. All reagents were obtained commercially and used without further purification. Solvents were purchased over molecular sieves (Fluka) and were used directly without further purification unless otherwise stated. All reactions were conducted under the rigorous exclusion of air and moisture. NMR spectroscopy: Bruker DPX300, DPX400, DRX500 with the solvent peak as internal standard. Fast atom bombardment mass spectrometry (FAB MS): Concept 1H (Kratos), matrix: 3-nitrobenzyl alcohol (3-NBA). Flash chromatography: Merck silica gel G60 (230–400 mesh). Thin-layer chromatography: Merck precoated plates (silica gel 60 F_{254}). MALDI-ToF MS analysis of oligonucleotides was conducted by Metabion, Germany. Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR MS) (Bruker APEX) was performed at the Kekulé-Institut für Organische Chemie und Biochemie, Bonn University.

3′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-methoxymethylene thymidine (2a): After coevaporation and drying under vacuum overnight, nucleoside **1** (325 mg, 0.52 mmol) was dissolved in THF (6 mL). Sodium hydride (44.8 mg, 1.12 mmol) was added at 0 °C and the reaction mixture was stirred for 30 min. Iodomethane (162 μL, 2.6 mmol) was added and stirring was continued for 10 h at 0 °C. The reaction was quenched by the addition of methanol (2 mL) and then allowed to warm up to room temperature. Saturated aqueous sodium bicarbonate solution was added and the aqueous phase was extracted with dichloromethane. The combined organic phase was dried ($MgSO_4$) and concentrated in a vacuum. Purification of the residue by flash column chromatography (SiO_2, ethyl acetate/cyclohexane 1:4–1:1) furnished **2a** as a white foam (250 mg, 0.39 mmol, 75 %); R_f = 0.64 (ethyl acetate/cyclohexane 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.03 (s, 3H; $SiCH_3$), 0.07 (s, 3H; $SiCH_3$), 0.90 (s, 9H; $SiC(CH_3)_3$), 1.08 (s, 9H; $SiC(CH_3)_3$), 1.60 (d, ^4J = 1.3 Hz, 3H; CH_3-5), 2.13–2.20 (m, 1H; H-2′a), 2.26 (ddd, ^2J = 13.0 Hz, ^3J = 5.7 Hz, ^3J = 2.0 Hz, 1H; H-2′b), 3.29 (s, 3H; OCH_3), 3.43 (d, ^2J = 10.1 Hz, 1H; H-5′a), 3.51 (d, ^2J = 10.1 Hz, 1H; H-5′b), 3.80 (d, ^2J = 10.9 Hz, 1H; 4′-C-CH_2a), 3.85 (d, ^2J = 10.9 Hz, 1H; 4′-C-CH_2b), 4.54 (dd, ^3J = 5.6 Hz, ^3J = 2.0 Hz, 1H; H-3′), 6.36 (dd, ^3J = 8.5 Hz, ^3J = 5.7 Hz, 1H; H-1′), 7.35–7.67 (m, 11H; Ar, H-6), 8.06 ppm (br s, 1H; NH); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = −5.0 ($SiCH_3$), −4.5 ($SiCH_3$), 12.2 (CH_3), 18.3 ($SiC(CH_3)_3$), 19.6 ($SiC(CH_3)_3$), 25.9 ($SiC(CH_3)_3$), 27.2 ($SiC(CH_3)_3$), 41.9 (C-2′), 59.7 (OCH_3), 66.0 (C-5′), 73.0 (4′-C-CH_2), 73.5 (C-3′), 84.7 (C-1′), 89.3 (C-4′), 111.1 (C-5), 128.17, 128.18, 130.27, 130.34, 132.7, 133.2, 135.64, 135.69, 135.8 (Ar, C-6), 150.3 (C-2), 163.6 ppm (C-4); FAB MS (3-NBA matrix): *m*/*z*: 639.3 $[M+H]^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-methoxymethylene thymidine (3a): A 1 M solution of TBAF (0.43 mL, 0.43 mmol) was added at 0 °C to a solution of **2a** (123 mg, 0.19 mmol) dissolved in THF (4 mL), and the cooled solution was stirred for 30 min. The reaction mixture was allowed to warm up to room temperature and stirring was continued for 3.5 h. A small amount of silica was added and the mixture was evaporated to dryness. The impregnated silica was coevaporated with toluene and subjected to column chromatography (SiO_2, cyclohexane/ethyl acetate 1:9–ethyl acetate/methanol 9:1) to yield the desired alcohol as a white foam (54.7 mg, 0.19 mmol, 99 %); R_f = 0.48 (ethyl acetate/methanol 9:1); ^{1}H NMR (400 MHz, CD_3OD): δ = 1.87 (d, ^4J = 1.1 Hz, 3H; CH_3-5), 2.36 (m, 2H; H-2′a, H-2′b), 3.37 (s, 3H; OCH_3), 3.55 (s, 2H; H-5′a, H-5′b), 3.80 (s, 2H; 4′-C-CH_2a, 4′-C-CH_2b), 4.48 (t, ^3J = 5.3 Hz, 1H; H-3′), 6.30 (dd, ^3J = ^3J = 6.7 Hz, 1H; H-1′), 7.81 ppm (d, ^4J = 1.1 Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, CD_3OD): δ = 12.6 (CH_3), 41.6 (C-2′), 59.9 (OCH_3), 64.6 (C-5′), 73.1 (4′-C-CH_2), 73.8 (C-3′), 86.1 (C-1′), 90.1 (C-4′), 111.7 (C-5), 138.4 (C-6), 152.6 (C-2), 166.4 ppm (C-4); FAB MS (3-NBA matrix): *m*/*z*: 287.1 $[M+H]^+$. The alcohol (53.7 mg, 0.19 mmol) was dissolved in pyridine (1 mL), 4,4′-dimethoxytrityl chloride (DMTCl) and a catalytic amount of 4-(dimethylamino)pyridine (DMAP) were added at 0 °C, and the cooled solution was stirred for 30 min. The reaction mixture was then allowed to warm up to room temperature and stirring was continued for 4 h. The reaction was quenched by the addition of methanol (1.5 mL) and stirring was continued for 30 min. The solvent was removed in a vacuum and the residue was purified by flash column chromatography (SiO_2, cyclohexane/ethyl acetate 3:7+1 % Et_3N). Compound **3a** was isolated as a white foam (60 mg, 0.10 mmol, 54 %); R_f = 0.28 (cyclohexane/ethyl acetate 3:7); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.49 (s, 3H; CH_3-5), 2.37–2.51 (m, 2H; H-2′a, H-2′b), 3.34 (d, ^2J = 9.9 Hz, 1H; H-5′a), 3.39 (s, 3H; OCH_3), 3.42 (d, ^2J = 9.9 Hz, 1H; H-5′b), 3.60 (d, ^2J = 10.0 Hz, 1H; 4′-C-CH_2a), 3.65 (d, ^2J = 10.0 Hz, 1H; 4′-C-CH_2b), 3.84 (s, 6H; OCH_3), 4.66 (dd, ^3J = 6.6 Hz, ^3J = 4.2 Hz, 1H; H-3′), 6.39 (dd, ^3J = ^3J = 6.6 Hz, 1H; H-1′), 6.91–7.50 (m, 13H; Ar), 7.63 ppm (s, 1H; H-6); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 12.4 (CH_3), 41.9 (C-2′), 55.9 (OCH_3), 59.9 (OCH_3), 66.5 (C-5′), 73.6 (4′-C-CH_2), 74.3 (C-3′), 86.1 (C-1′), 88.4 (C-4′), 89.7 (CAr_3), 114.3 (C-5), 128.2, 129.0, 129.6, 131.59, 131.6 (Ar), 137.0 (C-6), 137.1, 137.7 (Ar), 146.2 (C-2), 160.5 ppm (C-4); FAB MS (3-NBA matrix): *m*/*z*: 588.1 $[M+H]^+$, 303.1 $[DMT^+]$.

4′-*C*-Benzyloxymethylen-3′-*O*-*tert*-butyldimethylsilyl-5′-*O*-*tert*-butyldiphenylsilyl thymidine (2b): Sodium hydride (60 %, 21 mg, 0.53 mmol) was added at 0 °C to a solution of nucleoside **1** (109 mg, 0.17 mmol) dissolved in THF (2 mL), and the suspension was stirred for 30 min. At −60 °C benzyl bromide (104 μL, 0.88 mmol) and a catalytic amount of sodium iodide were added and stirring was continued for 1 h. The reaction mixture was then allowed to warm up to 0 °C under continual stirring for 2 h, after which the reaction was quenched by the addition of methanol (2 mL). After 30 min the solvent was evaporated, and the acquired residue was dissolved in dichloromethane and then poured onto saturated aqueous sodium bicarbonate solution. The aqueous layer was extracted with dichloromethane and the combined organic phase was dried over $MgSO_4$. Purification of the residue by flash column chromatography (SiO_2, cyclohexane/ethyl acetate 3:7) yielded **2b** (49.8 mg, 0.07 mmol, 40 %) as a white foam; R_f = 0.33 (cyclohexane/ethyl acetate 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ = −0.06 (s, 3H; $SiCH_3$), 0.02 (s, 3H; $SiCH_3$), 0.84 (s, 9H; $SiC(CH_3)_3$), 1.07 (s, 3H; $SiC(CH_3)_3$), 1.62 (d, ^4J = 1.1 Hz, 3H; CH_3-5), 2.15 (m, 1H; H-2′a), 2.26 (ddd, ^2J = 13.0 Hz, ^3J = 5.7 Hz, ^3J = 2.5 Hz, 1H; H-2′b), 3.49 (d, ^2J = 10.2 Hz, 1H; H-5′a), 3.56 (d, ^2J = 10.2 Hz, 1H; H-5′b), 3.81 (d, ^2J = 11.1 Hz, 1H; 4′-C-CH_2a), 3.87 (d, ^2J = 11.1 Hz, 1H; 4′-C-CH_2b), 4.42 (d, ^2J = 12.1 Hz, 1H; CH_2Ph), 4.43 (dd, ^3J = 8.1 Hz, ^3J = 5.7 Hz, 1H; H-3′), 4.53 (d, ^2J = 12.1 Hz, 1H; CH_2Ph), 6.36 (dd, ^3J = 8.3 Hz, ^3J = 5.7 Hz, 1H; H-1′), 7.15–7.66 (m, 16H; Ar, C-6), 8.18 ppm (br s, 1H; NH); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = −5.6 ($SiCH_3$), −4.6 ($SiCH_3$), 12.3 (CH_3), 18.2 ($SiC(CH_3)_3$), 19.6 ($SiC(CH_3)_3$), 25.9 ($SiC(CH_3)_3$), 27.2 ($SiC(CH_3)_3$), 41.8 (C-2′), 66.0 (C-5′), 70.4 (4′-C-CH_2), 73.4 (C-3′), 73.9 (CH_2Ph), 84.7 (C-1′), 89.4 (C-4′), 111.1 (C-5), 127.75. 127.8, 127.9, 128.07, 128.1, 128.2, 128.5, 128.7, 130.28, 130.3, 132.6, 133.1, 135.56, 135.6, 135.75, 135.8, 138.1 (Ar, C-6), 150.3 (C-2), 163.7 ppm (C-4); FAB MS (3-NBA matrix): *m*/*z*: 737.4 $[M+Na]^+$, 715.4 $[M+H]^+$, 657.3 $[M-t\text{Bu}]^+$, 607.2 $[M-\text{BnO}]^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-benzyloxymethylene thymidine (3b): A 1 M solution of TBAF (140 μL, 0.14 mmol) was added to a solution of nucleoside **2b** (44.6 mg, 0.06 mmol) in THF (2 mL) and the mixture was stirred at room temperature for 7 h. The solvent was removed and the residue was purified by column chromatography (SiO_2, ethyl acetate) to yield the alcohol as a pale yellow solid (18.6 mg, 0.05 mmol, 83 %); R_f = 0.24 (ethyl acetate); ^{1}H NMR (400 MHz, CD_3OD): δ = 1.95 (d, ^4J = 1.1 Hz, 3H; CH_3-5), 2.36–2.48 (m, 2H; H-2′a, H-2′b), 3.70 (d, ^2J = 10.1 Hz, 1H; H-5′a), 3.74 (d, ^2J = 10.1 Hz, 1H; H-5′b), 3.78 (d, ^2J = 11.7 Hz, 1H; 4′-C-CH_2a), 3.82 (d, ^2J = 11.7 Hz, 1H; 4′-C-CH_2b), 4.57–4.67 (m, 3H; H-3′, CH_2Ph), 6.40 (dd, ^3J = ^3J = 6.6 Hz, 1H; H-1′), 7.31–7.45 (m, 5H; Ar), 7.86 ppm (d, ^4J = 1.1 Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, CD_3OD): δ = 12.8 (CH_3), 41.7 (C-2′), 64.7 (C-5′), 71.5 (4′-C-CH_2), 73.0

(C-3′), 74.9 (CH_2Ph), 86.2 (C-1′), 90.1 (C-4′), 111.8 (C-5), 128.8, 128.9, 129.5, 129.6, 138.3, 139.8 (Ar, C-6), 154.0 (C-2), 168.5 ppm (C-4); FAB MS (3-NBA matrix): *m/z*: 363.1 $[M+H]^+$. The alcohol (18.5 mg, 0.05 mmol) was dissolved in 0.5 mL pyridine, DMTCl and a catalytic amount of DMAP were added, and the solution was stirred at room temperature for 19 h. The reaction was then quenched by the addition of methanol (1 mL) and stirring was continued for 30 min. After evaporation, the residue was subjected to flash column chromatography (SiO_2, cyclohexane/ethyl acetate 1:1+1% triethylamine) to furnish **3b** as a yellowish foam (11.4 mg, 0.02 mmol, 33%); R_f=0.64 (ethyl acetate); ^{1}H NMR (300 MHz, CD_3OD): δ=1.55 (d, ^4J=0.9 Hz, 3H; CH_3-5), 2.33 (m, 1H; H-2′a), 2.46 (ddd, ^2J=13.5 Hz, ^3J=6.5 Hz, ^3J=3.8 Hz, 1H; H-2′b), 3.72 (d, ^2J=10.2 Hz, 1H; 4′-C-CH_2a), 3.81 (d, ^2J=10.2 Hz, 1H; 4′-C-CH_2b), 4.55 (d, ^2J=12.2 Hz, 1H; CH_2Ph), 4.62 (d, ^2J=12.2 Hz, 1H; CH_2Ph), 4.64 (m, 1H; H-3′), 6.47 (dd, ^3J=^3J=6.5 Hz, 1H; H-1′), 6.88–7.53 (m, 18H; Ar), 7.55 ppm (d, ^4J=0.9 Hz, 1H; H-6); ^{13}C NMR (75.5 MHz, CD_3OD): δ=13.1 (CH_3), 42.0 (C-2′), 55.9 (OCH_3), 66.7 (C-5′), 71.8 (4′-C-CH_2), 73.8 (C-3′), 74.7 (CH_2Ph), 86.2 (C-1′), 88.4 (CAr_3), 89.6 (C-4′), 112.0 (C-5), 114.3, 128.2, 128.7, 128.9, 129.0, 129.5, 129.6, 130.6, 131.0, 131.6, 131.62, 137.0, 137.1, 137.2, 139.8 (Ar), 146.3 (C-2), 160.4, 160.5 ppm (Ar, C-4); FAB MS (3-NBA matrix): *m/z*: 664.3 $[M^+]$, 303.1 $[DMT^+]$.

3′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-phenoxymethylene thymidine (2c): Pyridine (70 μL, 0.89 mmol) was added at 0°C to a solution of nucleoside **1** (212 mg, 0.28 mmol) in dichloromethane (2 mL). Trifluoromethanesulfonic anhydride (69.5 μL, 0.43 mmol) was added drop-wise and stirring was continued at 0°C for 1.5 h, after which the reaction was quenched by the addition of saturated $NaHCO_3$ solution (1 mL). Additional dichloromethane was added and the organic phase was washed with saturated $NaHCO_3$ and NaCl solution. The organic phase was dried ($MgSO_4$) and concentrated to give a dark-red residue, which was used directly in the next reaction step without further purification. Sodium hydride (113 mg, 2.84 mmol) was added at −78°C to a solution of phenol (267 mg, 2.84 mmol) dissolved in DMF (3 mL), and the reaction mixture was stirred for 30 min as it warmed up to 0°C. The crude residue was then added and stirred at 0°C for 1 h. Next, the mixture was allowed to warm up to room temperature and then heated to 50°C for 1.5 h, after which the mixture was poured onto saturated aqueous sodium bicarbonate solution. The aqueous phase was extracted with dichloromethane, and the combined organic phase was dried ($MgSO_4$) and concentrated. Purification by flash column chromatography (SiO_2, cyclohexane/ethyl acetate 4:1) yielded **2c** as a foam (98.4 mg, 0.14 mmol, 50%); R_f=0.61 (ethyl acetate/cyclohexane 3:7); ^{1}H NMR (500 MHz, $CDCl_3$): δ=0.07 (s, 3H; $SiCH_3$), 0.17 (s, 3H; $SiCH_3$), 0.94 (s, 9H; $SiC(CH_3)_3$), 1.16 (s, 9H; $SiC(CH_3)_3$), 1.76 (d, ^4J=1.0 Hz, 3H; CH_3-5), 2.36 (m, 1H; H-2′a), 2.42 (ddd, ^2J=13.1 Hz, ^3J=5.6 Hz, ^3J=1.7 Hz, 1H; H-2′b), 4.02 (d, ^2J=11.2 Hz, 1H; H-5′a), 4.07 (d, ^2J=11.2 Hz, 1H; H-5′b), 4.10 (d, ^2J=9.4 Hz, 1H; 4′-C-CH_2a), 4.16 (d, ^2J=9.4 Hz, 1H; 4′-C-CH_2b), 4.68 (d, ^3J=5.2 Hz, ^3J=1.7 Hz, 1H; H-3′), 6.52 (dd, ^3J=8.7 Hz, ^3J=5.6 Hz, 1H; H-1′), 6.86–7.79 (m, 15H; Ar), 7.57 (d, ^4J=1.0 Hz, 1H; H-6), 9.24 ppm (br s, 1H; NH); ^{13}C NMR (125.8 MHz, $CDCl_3$): δ=−5.0 ($SiCH_3$), −4.6 ($SiCH_3$), 12.4 (CH_3), 18.1 ($SiC(CH_3)_3$), 19.6 ($SiC(CH_3)_3$), 25.9 ($SiC(CH_3)_3$), 27.2 ($SiC(CH_3)_3$), 41.9 (C-2′), 66.0 (C-5′), 67.3 (4′-C-CH_2), 73.6 (C-3′), 85.0 (C-1′), 89.1 (C-4′), 111.3 (C-5), 114.4, 121.1, 127.9, 128.0, 128.20, 128.22, 129.0, 130.02, 130.04, 130.4, 131.1, 132.4, 132.9, 135.6 (Ar), 135.8, 135.9 (Ar, C-6), 150.5 (C-2), 158.6 (Ar), 164.3 ppm (C-4); FAB MS (3-NBA matrix): *m/z*: 723.2 $[M+Na]^+$, 701.4 $[M+H]^+$, 643.2 $[M-t\text{Bu}+H]^+$, 623.1 $[M-\text{Ph}+H]^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-phenoxymethylene thymidine (3c): A 1 M solution of TBAF (250 μL, 0.25 mmol) was added to a solution of compound **2c** (79.6 mg, 0.11 mmol) in THF (2 mL) and the mixture was stirred at room temperature for 1 h. The solvent was then evaporated under reduced pressure and the residue was purified by column chromatography (SiO_2, dichloromethane/methanol 9:1). The resulting alcohol was isolated as a yellowish solid (36.9 mg, 0.11 mmol, 93%); R_f=0.24 (cyclohexane/ethyl acetate 1:9); ^{1}H NMR (400 MHz, CD_3OD): δ=1.94 (d, ^4J=1.2 Hz, 3H; CH_3-5), 2.43–2.55 (m, 2H; H-2a′, H-2b′), 3.88 (d, ^2J=11.6 Hz, 1H; H-5′a), 3.91 (d, ^2J=11.6 Hz, 1H; H-5′b), 4.17 (d, ^2J=10.0 Hz, 1H; 4′-CH_2a), 4.21 (d, ^2J=10.0 Hz, 1H; 4′-C-CH_2b), 4.66 (dd, ^3J=6.5 Hz, ^3J=4.6 Hz, 1H; H-3′), 6.44 (dd, ^3J=^3J=6.7 Hz, 1H; H-1′), 6.95–7.38 (m, 5H; Ar), 7.86 ppm (d, ^4J=1.2 Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, CD_3OD): δ=12.7 (CH_3), 41.7 (C-2′), 64.5 (C-5′), 69.1 (4′-C-CH_2), 73.0 (C-3′), 86.4 (C-1′), 89.7 (C-4′), 111.8 (C-5), 115.8, 122.1, 130.6 (Ar), 138.4 (C-6), 153.4 (C-2), 160.6 (Ar), 167.6 ppm (C-4); FAB MS (3-NBA matrix): *m/z*: 349.1 $[M+H]^+$. The alcohol (35 mg, 0.10 mmol) was dissolved in pyridine (0.5 mL), and DMTCl (86.3 mg, 0.26 mmol) and a catalytic amount of DMAP were added to the solution. After being stirred for 17 h at room temperature, methanol (0.5 mL) was added and stirring was continued for 30 min. The solvent was evaporated in a vacuum and the resulting residue was subjected to column chromatography (SiO_2, cyclohexane/ethyl acetate 1:1+1% triethylamine–ethyl acetate+1% triethylamine) to furnish **3c** as a slightly yellow foam (38.4 mg, 0.06 mmol, 59%); R_f=0.44 (cyclohexane/ethyl acetate 3:7); ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.59 (d, ^4J=1.1 Hz, 3H; CH_3-5), 2.35 (m, 1H; H-2a′), 2.55 (ddd, ^2J=13.6 Hz, ^3J=6.6 Hz, ^3J=3.8 Hz, 1H; H-2′b), 3.39 (d, ^2J=9.9 Hz, 1H; H-5a′), 3.55 (d, ^2J=9.9 Hz, 1H; H-5′b), 3.82 (s, 6H; OCH_3), 4.25 (d, ^2J=10.0 Hz, 1H; 4′-C-CH_2a), 4.30 (d, ^2J=10.0 Hz, 1H; 4′-C-CH_2b), 4.70 (dd, ^3J=6.6 Hz, ^3J=3.8 Hz, 1H; H-3′), 6.46 (dd, ^3J=^3J=6.6 Hz, 1H; H-1′), 6.86–7.50 (m, 18H; Ar), 7.52 ppm (d, ^4J=1.1 Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ=12.6 (CH_3), 41.6 (C-2′) 55.7 (OCH_3), 66.0 (C-5′), 68.9 (4′-C-CH_2), 73.5 (C-3′), 86.4 (C-1′), 88.2 (C-4′), 89.2 ($CAr3$), 111.8 (C-5), 114.2, 115.7, 121.9, 128.0, 128.9, 129.4, 130.5, 131.4, 131.5, 136.8, 137.0, 137.2, 146.1 (Ar), 154.2 (C-2), 160.28, 160.3, 160.31 ppm (Ar, C-4); FAB MS (3-NBA matrix): *m/z*: 673.3 $[M+Na]^+$, 650.4 $[M^+]$, 303.1 $[DMT^+]$.

3-*O*-Benzyl-5-*O*-*tert*-butyldiphenylsilyl-4-*C*-ethoxymethylene-1,2-isopropylidene-β-D-*ribo*-pentofuranose (9): Compound **8** (725 mg, 1.32 mmol) was dissolved in DMF (5 mL) and the solution was cooled to 0°C. Sodium hydride (82.8 mg, 2.07 mmol) was added and the reaction mixture was stirred for 30 min at low temperature. The mixture was then cooled to −20°C, iodoethane (1.07 mL, 13.24 mmol) was added, and stirring was continued for 3 h. The reaction mixture was allowed to warm up to 0°C and stirring was continued for 1 h. The reaction was quenched by the addition of methanol (5 mL) and then warmed up to room temperature within 45 min. Saturated $NaHCO_3$ solution was added and the aqueous phase was extracted with dichloromethane. The combined organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. Column chromatography (SiO_2, ethyl acetate/cyclohexane 1:9) furnished a white solid (**9**, 479 mg, 63%); R_f=0.51 (ethyl acetate/cyclohexane 1:4); ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.98 (s, 9H; $SiC(CH_3)_3$), 1.07 (t, ^3J=7.0 Hz, 3H; CH_3-ethyl), 1.20 (s, 6H; CH_3-acetonide), 3.30–3.48 (m, 2H; CH_2-ethyl), 3.47 (d, ^2J=10.2 Hz, 1H; H-5a), 3.59 (d, ^2J=10.2 Hz, 1H; H-5b), 3.96 (d, ^2J=11.1 Hz, 1H; 4-C-CH_2a), 4.02 (d, ^2J=11.1 Hz, 1H; 4-C-CH_2b), 4.08 (d, ^3J=5.4 Hz, 1H; H-3), 4.46 (d, ^2J=12.3 Hz, 1H; CH_2Ph), 4.51 (dd, ^3J=5.4 Hz, ^3J=4.0 Hz, 1H; H-2), 4.65 (d, ^2J=12.3 Hz, 1H; CH_2Ph), 5.68 (d, ^3J=4.0 Hz, 1H; H-1), 7.16–7.67 ppm (m, 15H; Ar); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ=15.4 (CH_3), 19.6 ($SiC(CH_3)_3$), 26.6 (CH_3), 26.8 (CH_3), 27.1 ($SiC(CH_3)_3$), 64.9 (C-5), 67.2 (CH_2), 72.5 (4-C-CH_2), 72.6 (CH_2Ph), 78.4 (C-3), 79.9 (C-2), 87.9 (C-4), 104.5 (C-1), 113.5 ($O_2C(CH_3)_2$), 127.7, 127.8, 127.9, 128.0, 128.2, 128.5, 128.7, 129.0, 129.7, 131.1, 133.7, 134.0, 135.0, 135.8, 135.9, 136.0, 136.1, 138.3 ppm (Ar); FAB MS (3-NBA matrix): *m/z*: 577.3 $[M+H]^+$, 561.2 $[M-\text{Me}]^+$, 519.2 $[M-t\text{Bu}]^+$.

1-(2′-*O*-Acetyl-3′-*O*-benzyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-ethoxymethylene-β-D-*ribo*-pentofuranosyl)thymine (10): Compound **9** (400 mg, 0.69 mmol) was dissolved in a mixture of 80% acetic acid (10 mL) and trifluoroacetic acid (500 μL), and the resulting solution was stirred at room temperature for 4 h. The solvent was then removed under reduced pressure and the remaining residue was coevaporated with toluene. The residue was stirred with acetic anhydride (0.7 mL, 7.41 mmol) and a catalytic amount of DMAP in pyridine (5 mL) for 18 h at ambient temperature, and the reaction mixture was evaporated to dryness. The resulting residue was diluted by the addition of dichloromethane and then poured onto aqueous $NaHCO_3$. The aqueous phase was extracted with dichloromethane, and the combined organic phase was dried ($MgSO_4$) and concentrated. Purification by column chromatography (SiO_2, ethyl acetate/cyclohexane 1:4) yielded the desired bis-acetate as a white foam (150.8 mg, 0.24 mmol, 35%); R_f=0.42 (ethyl acetate/cyclohexane 1:4);

^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.97$ (s, 9H; $SiC(CH_3)_3$), 1.13 (t, $^3J = 7.0$ Hz, 3H; CH_3-ethyl), 1.73 (s, 3H; $OCOCH_3$), 1.98 (s, 3H; $OCOCH_3$), 3.34–3.69 (m, 5H; 4-C-CH_2a, 4-C-CH_2b, CH_2-ethyl, H-5a), 3.73 (d, $^2J = 9.9$ Hz, 1H; H-5b), 3.76 (d, $^2J = 11.0$ Hz, 1H; CH_2Ph), 3.87 (d, $^2J = 11.0$ Hz, 1H; CH_2Ph), 4.27 (d, $^3J = 5.1$ Hz, 1H; H-3), 5.18 (dd, $^3J = 5.1$ Hz, $^3J = 1.2$ Hz, 1H; H-2), 6.00 (d, $^3J = 1.2$ Hz, 1H; H-1), 7.10–7.66 ppm (m, 15H; Ar); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 15.4$ (CH_3), 19.5 ($SiC(CH_3)_3$), 20.8 ($OCOCH_3$), 21.4 ($OCOCH_3$), 27.0 ($SiC(CH_3)_3$), 64.3 (CH_2), 67.3 (C-5), 71.7 (4-C-CH_2), 73.6 (CH_2Ph), 75.2 (C-3), 78.9 (C-2), 87.4 (C-4), 97.9 (C-1), 127.4, 127.7, 127.8, 128.0, 128.5, 129.6, 129.7, 131.0, 133.5, 134.1, 135.78, 135.79, 135.8, 136.2, 138.0 (Ar), 169.4, 169.9 ppm ($OCOCH_3$); FAB MS (3-NBA matrix): m/z: 619.2 [M^+], 561.2 [$M-$OAc]$^+$. The bis-acetate (143 mg, 0.23 mmol), thymine (58.9 mg, 0.47 mmol), and *N*,*O*-bis(trimethylsilyl)acetamide (BSA, 300 μL, 1.23 mmol) were suspended in acetonitrile (2 mL). The suspension was stirred at 60 °C for 30 min until it became completely soluble. The solution was then cooled to 0 °C, after which trimethylsilyl trifluoromethane sulfonate (80 μL, 0.44 mmol) was added. The reaction mixture was refluxed for 1 h and saturated aqueous $NaHCO_3$ (10 mL) was added at ambient temperature. The organic phase was extracted with dichloromethane, then dried ($MgSO_4$) and concentrated. Purification by column chromatography (SiO_2, ethyl acetate/cyclohexane 2:3) afforded a white solid (**10**, 144.2 mg, 0.21 mmol, 91 %); $R_f = 0.30$ (ethyl acetate/cyclohexane 2:3); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.99$ (s, 9H; $SiC(CH_3)_3$), 1.17 (t, $^3J = 7.0$ Hz, 3H; CH_3-ethyl), 1.84 (d, $^4J = 1.1$ Hz, 3H; CH_3-5), 1.86 (s, 3H; $OCOCH_3$), 3.39–3.52 (m, 2H; CH_2-ethyl), 3.52 (d, $^2J = 10.3$ Hz, 1H; 4′-C-CH_2a), 3.63 (d, $^2J = 10.3$ Hz, 1H; 4′-C-CH_2b), 3.65 (d, $^2J = 11.0$ Hz, 1H; H-5′a), 3.86 (d, $^2J = 11.0$ Hz, 1H; H-5′b), 4.29 (d, $^3J = 5.7$ Hz, 1H; H-3′), 4.47 (d, $^2J = 11.5$ Hz, 1H; CH_2Ph), 4.50 (d, $^2J = 11.5$ Hz, 1H; CH_2Ph), 5.26 (dd, $^3J = 6.2$ Hz, $^3J = 5.7$ Hz, 1H; H-2′), 6.08 (d, $^3J = 6.2$ Hz, 1H; H-1′), 7.12–7.64 (m, 16H; Ar, H-6), 8.63 ppm (br s, 1H; NH); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 12.8$ (CH_3), 15.4 (CH_3), 19.5 ($SiC(CH_3)_3$), 20.8 ($COCH_3$), 27.1 ($SiC(CH_3)_3$), 64.2 (C-5′), 67.3 (CH_2), 72.6 (4′-C-CH_2), 74.8 (CH_2Ph), 75.3 (C-3′), 77.9 (C-2′), 85.9 (C-1′), 88.2 (C-4′), 111.3 (C-5), 127.9, 127.92, 127.97, 128.0, 128.2, 128.22, 128.56, 128.6, 130.0, 130.1, 133.0, 133.3, 135.6, 135.8, 135.9, 135.97, 136.0, 137.8 (Ar, C-6), 150.6 (C-2), 163.8 (C-4), 170.3 ppm ($COCH_3$); FAB MS (3-NBA matrix): m/z: 687.4 [M+H]$^+$, 629.2 [$M-t$Bu]$^+$, 609.3 [$M-$Ph]$^+$, 561.2 [$M-$thymine]$^+$.

1-(3′-*O*-Benzyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-ethoxymethylene-β-D-*ribo*-pentofuranosyl)thymine (11): A solution of nucleoside **10** (132.8 mg, 0.19 mmol) and sodium methoxide (22 mg, 0.41 mmol) in methanol (2 mL) was stirred at room temperature for 1 h, after which the solvent was removed in a vacuum. The residue was dissolved in dichloromethane, poured onto saturated aqueous NH_4Cl, and extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$ and concentrated. Chromatography over silica gel (SiO_2, ethyl acetate/cyclohexane 2:3–1:1) yielded the desired alcohol as a white foam (117.1 mg, 0.18 mmol, 94 %); $R_f = 0.39$ (ethyl acetate/cyclohexane 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.00$ (s, 9H; $SiC(CH_3)_3$), 1.12 (d, $^3J = 7.0$ Hz, 3H; CH_3-ethyl), 1.83 (d, $^4J = 1.1$ Hz, 3H; CH_3-5), 3.37–3.43 (m, 2H; CH_2-ethyl), 3.41 (d, $^2J = 10.2$ Hz, 1H; 4′-C-CH_2a), 3.46 (d, $^2J = 10.2$ Hz, 1H; 4′-C-CH_2b), 3.68 (d, $^2J = 10.9$ Hz, 1H; H-5′a), 3.75 (d, $^2J = 10.9$ Hz, 1H; H-5′b), 4.19 (d, $^3J = 6.1$ Hz, 1H; H-3′), 4.33 (m, 1H; H-2′), 4.60 (d, $^2J = 11.1$ Hz, 1H; CH_2Ph), 4.67 (d, $^2J = 11.1$ Hz, 1H; CH_2Ph), 5.88 (d, $^3J = 4.9$ Hz, 1H; H-1′), 7.19–7.64 (m, 15H; Ar), 7.42 ppm (d, $^4J = 1.1$ Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 12.8$ (CH_3), 15.3(CH_3), 19.3 ($SiC(CH_3)_3$), 27.1 ($SiC(CH_3)_3$), 64.4 (C-5), 67.3 (CH_2), 72.6 (4′-C-CH_2), 74.4 (C-2′), 74.9 (CH_2Ph), 78.8 (C-3′), 88.3 (C-4′), 91.1 (C-1′), 110.9 (C-5), 127.9, 128.0, 128.06, 128.1, 128.18, 128.2, 128.3, 128.66, 128.7, 130.1, 132.5, 132.6, 135.6, 135.8, 135.86, 135.9, 136.8, 137.6, 137.8 (Ar, C-6), 150.7 (C-2), 163.9 ppm (C-4); FAB MS (3-NBA matrix): m/z: 645.4 [M+H]$^+$, 587.1 [$M-t$Bu]$^+$, 567.3 [$M-$Ph]$^+$. *O*-Phenyl chlorothionoformate (30 μL, 0.22 mmol) was added drop-wise to a solution of the alcohol (117 mg, 0.18 mmol) and DMAP (84.2 mg, 0.69 mmol) in acetonitrile (2 mL), and the resulting solution was stirred at ambient temperature for 1 h. The mixture was then diluted with dichloromethane and poured onto saturated aqueous $KHSO_4$. The organic phase was separated by using dichloromethane, the extracts were dried over $MgSO_4$ and then concentrated. Purification by column chromatography (SiO_2, ethyl acetate/cyclohexane 2:8) yielded the thiocarbonate as a white foam (119.2 mg, 0.15 mmol, 85 %); $R_f = 0.69$ (ethyl acetate/cyclohexane 1:1). A solution of thiocarbonate (113.5 mg, 0.15 mmol) in toluene (1 mL) was added drop-wise to a solution of 2,2′-azobisisobutyronitrile (AIBN, 7 mg, 0.04 mmol) and tri-*n*-butyltin hydride (120 μL, 0.45 mmol) in anhydrous toluene (0.5 mL) at 85 °C, and the reaction mixture was heated to reflux for 3 h. The solvent was removed under reduced pressure and the residue was purified by chromatography over silica gel (SiO_2, ethyl acetate/cyclohexane 3:7). Compound **11** was obtained as a white foam (63.4 mg, 0.10 mmol, 69 %); $R_f = 0.48$ (ethyl acetate/cyclohexane 1:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.05$ (s, 9H; $SiC(CH_3)_3$), 1.21 (t, $^3J = 7.0$ Hz, 3H;, CH_3-ethyl), 1.91 (d, $^4J = 1.0$ Hz, 3H; CH_3-5), 2.20 (m, 1H; H-2′a), 2.60 (ddd, $^2J = 13.4$ Hz, $^3J = 6.5$ Hz, $^3J = 4.9$ Hz, 1H; H-2′b), 3.40–3.84 (m, 6H; 4′-C-CH_2a, 4′-C-CH_2b, H-5′a, H-5′b, CH_2-ethyl), 4.33 (dd, $^3J = 6.6$ Hz, $^3J = 4.9$ Hz, 1H; H-3′), 4.47 (d, $^2J = 11.8$ Hz, 1H; CH_2Ph), 4.60 (d, $^2J = 11.8$ Hz, 1H; CH_2Ph), 6.32 (dd, $^3J = {}^3J = 6.5$ Hz, 1H; H-1′), 7.22–7.67 (m, 15H; Ar), 7.71 (d, $^4J = 1.0$ Hz, 1H; H-6), 8.30 ppm (br s, 1H; NH); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 12.8$ (CH_3), 15.4 (CH_3), 19.4 ($SiC(CH_3)_3$), 27.1 ($SiC(CH_3)_3$), 38.8 (C-2′), 64.3 (C-5′), 67.3 (CH_2-ethyl), 72.2 (4′-C-CH_2), 72.6 (Bn), 78.8 (C-3′), 84.9 (C-1′), 88.8 (C-4′), 110.6 (C-5), 127.7, 127.8, 127.9, 127.94, 128.1, 128.6, 128.7, 129.9, 129.95, 133.1, 133.2, 135.6, 135.7, 135.8, 135.9, 136.6 (Ar, C-6), 138.0, 150.4 (C-2), 163.9 ppm (C-4); FAB MS (3-NBA matrix): m/z: 629.3 [M+H]$^+$, 571.2 [$M-t$Bu]$^+$.

1-(4′-*C*-Ethoxymethyl-β-D-*ribo*-pentofuranosyl)thymine (12): A 1 M solution of TBAF (220 μL, 0.22 mmol) was added to a solution of **11** (57.6 mg, 0.09 mmol) in 2 mL anhydrous THF and stirring was continued at ambient temperature for 1 day, after which the solvent was evaporated. Chromatography over silica (SiO_2, ethyl acetate/cyclohexane 8:2) furnished a colorless intermediate, which was used in the next reaction step. Palladium hydroxide on activated charcoal (20 %, 19.5 mg) was added to the resulting residue, which was then suspended in ethanol (1 mL). The mixture was degassed, flushed with argon, and placed under a hydrogen atmosphere. After stirring for 6 h at 60 °C the catalyst was filtered off by using celite, then washed with ethanol, and the filtrate was concentrated. Purification of the residue by flash column chromatography (SiO_2, ethyl acetate–methanol/ethyl acetate 1:9) yielded **12** as a colorless solid (19 mg, 0.06 mmol, 69 %); $R_f = 0.15$ (ethyl acetate); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 1.32$ (t, $^3J = 7.0$ Hz, 3H; CH_3-ethyl), 1.95 (d, $^4J = 1.2$ Hz, 3H; CH_3-5), 2.39–2.42 (m, 2H; H-2′a, H-2′b), 3.53–3.83 (m, 6H; 4′-C-CH_2a, 4′-C-CH_2b, H-5′a, H-5′b, CH_2-ethyl), 4.59 (dd, $^3J = 5.9$ Hz, $^3J = 4.5$ Hz, 1H; H-3′), 6.41 (dd, $^3J = {}^3J = 6.6$ Hz, 1H; H-1′), 7.86 ppm (d, $^4J = 1.2$ Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 12.9$ (CH_3), 15.7 (CH_3), 42.3 (C-2′), 63.8 (C-5′), 68.2 (CH_2-ethyl), 73.4 (4′-C-CH_2), 73.7 (C-3′), 86.2 (C-1′), 89.9 (C-4′), 111.5 (C-5), 138.0 (C-6), 153.7 (C-2), 168.0 ppm (C-4); FAB MS (3-NBA matrix): m/z: 340.4 [M+K]$^+$, 286.3 [$M-CH_3$]$^+$.

1-(5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-ethoxymethyl-β-D-*ribo*-pentofuranosyl)thymine (13): DMTCl (94.7 mg, 0.28 mmol) and a catalytic amount of DMAP were added to a solution of **12** (16.8 mg, 0.06 mmol) in anhydrous pyridine (1 mL). After being stirred at 50 °C for 4 h the reaction was quenched by the addition of methanol (2 mL), and stirring was continued for 30 min. The mixture was concentrated and purified by column chromatography (SiO_2, ethyl acetate/cyclohexane 8:2+1 % triethylamine–ethyl acetate+1 % triethylamine) to furnish **13** as a foam (8.6 mg, 0.014 mmol, 25 %); $R_f = 0.55$ (ethyl acetate); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 1.32$ (t, $^3J = 7.6$ Hz, 3H; CH_3-ethyl), 1.97 (d, $^4J = 1.1$ Hz, 3H; CH_3-5), 2.25–2.40 (m, 2H; H-2′a, H-2′b), 3.24 (d, $^2J = 10.0$ Hz, 1H; H-5′a), 3.48 (d, $^2J = 10.0$ Hz, 1H; H-5′b), 3.56–3.69 (m, 4H; 4′-C-CH_2a, 4′-C-CH_2b, CH_2-ethyl), 3.85 (s, 6H; OCH_3), 4.54 (dd, $^3J = 5.4$ Hz, $^3J = 4.9$ Hz, 1H; H-3′), 6.46 (dd, $^3J = {}^3J = 7.0$ Hz, 1H; H-1′), 6.91–8.00 ppm (m, 14H; Ar, H-6); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta = 13.5$ (CH_3), 15.8 (CH_3), 42.1 (C-2′), 55.8 (OCH_3), 64.9 (C-5′), 68.2 (CH_2), 73.5, 73.7 (C-3′, 4′-C-CH_2), 85.9 (C-1′), 87.8 (C-4′), 89.7 (CAr_3), 111.8 (C-5), 114.2, 114.3, 127.8, 127.83, 128.8, 128.9, 129.2, 129.6, 130.8, 131.5, 131.6, 137.4, 137.5, 137.6, 137.7 (Ar, C-6), 146.7 (C-2), 160.3 ppm (C-4); FAB MS (3-NBA matrix): m/z: 603.3 [M+H]$^+$, 303.2 [DMT$^+$].

3′-*O*-*tert*-Butyldimethylsilyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-carboxymethyl thymidine (16): Nucleoside **1** (501 mg, 0.80 mmol), powdered mo-

lecular sieve (4 Å, 626 mg), and pyridinium dichromate (3 g, 7.97 mmol) were suspended in anhydrous DMF (6 mL), and the mixture was stirred at room temperature for 3 h. Water (6 mL) and acetic acid (4 mL) were added and stirring was continued for 30 min. The reaction mixture was then diluted with ethyl acetate, the precipitate was filtered off and the aqueous phase was extracted with ethyl acetate. The organic phase was washed with an aqueous solution of oxalic acid (1.78 g per 50 mL) and ammonium oxalate (2 g per 50 mL), and the aqueous phase was extracted with ethyl acetate. The combined organic phase was dried ($MgSO_4$) and concentrated in a vacuum. Column chromatography (SiO_2, cyclohexane/ethyl acetate 2:3+1% acetic acid) yielded the desired carboxylic acid (338 mg, 0.52 mmol, 65%) as a yellow foam; $R_f=0.81$ (cyclohexane/ethyl acetate 3:7); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.01$ (s, 3H; $SiCH_3$), 0.05 (s, 3H; $SiCH_3$), 0.84 (s, 9H; $SiC(CH_3)_3$), 1.08 (s, 9H; $SiC(CH_3)_3$), 1.52 (d, $^4J=1.1$ Hz, 3H; CH_3-5), 2.23–2.29 (m, 1H; H-2′a), 2.36 (ddd, $^2J=12.9$ Hz, $^3J=5.7$ Hz, $^3J=2.1$ Hz, 1H; H-2′b), 4.07 (d, $^2J=11.4$ Hz, 1H; H-5′a), 4.18 (d, $^2J=11.4$ Hz, 1H; H-5′b), 4.61 (dd, $^3J=5.5$ Hz, $^3J=2.1$ Hz, 1H; H-3′), 6.61 (dd, $^3J=8.5$ Hz, $^3J=5.7$ Hz, 1H; H-1′), 7.31–7.67 (m, 11H; Ar, H-6), 8.86 ppm (br s, 1H; NH); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta=-5.1$ ($SiCH_3$), −4.7 ($SiCH_3$), 12.1 (CH_3), 18.0 ($SiC(CH_3)_3$), 19.7 ($SiC(CH_3)_3$), 25.7 ($SiC(CH_3)_3$), 27.3 ($SiC(CH_3)_3$), 41.4 (C-2′), 65.8 (C-5′), 74.3 (C-3′), 86.2 (C-1′), 92.9 (C-4′), 111.6 (C-5), 127.8, 127.9, 128.2, 128.4, 129.9, 130.4, 130.6, 132.3, 132.8, 135.5, 135.7, 135.9, 136.0 (Ar, C-6), 150.3 (C-2), 164.1 (C-4), 172.3 ppm (CO_2H); FAB MS (3-NBA matrix): m/z: 661.2 $[M+Na]^+$, 639.3 $[M+H]^+$, 581.2 $[M-t\mathrm{Bu}]^+$, 561.2 $[M-\mathrm{Ph}]^+$. Anhydrous methanol (60 μL, 1.48 mmol) was added at 0°C to a solution of the carboxylic acid (87.8 mg, 0.14 mmol), EDC (166 mg, 0.86 mmol), and DMAP (50.2 mg, 0.41 mmol) in anhydrous dichloromethane (2 mL). The reaction mixture was allowed to warm up to room temperature and stirring was continued for 1 day. After being quenched with water (10 mL) the mixture was extracted with CH_2Cl_2, the organic phase was dried over magnesium sulfate, and then evaporated under reduced pressure. Purification by flash column chromatography (SiO_2, cyclohexane/ethyl acetate 4:1–3:2) furnished **16** as a white solid (60.8 mg, 0.09 mmol, 68%); $R_f=0.45$ (ethyl acetate/cyclohexane 2:3); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.01$ (s, 3H; $SiCH_3$), 0.05 (s, 3H; $SiCH_3$), 0.85 (s, 9H; $SiC(CH_3)$), 1.08 (s, 9H; $SiC(CH_3)_3$), 1.49 (d, $^4J=1.1$ Hz, 3H; CH_3-5), 2.20 (m, 1H; H-2′a), 2.37 (ddd, $^2J=13.1$ Hz, $^3J=6.0$ Hz, $^3J=2.8$ Hz, 1H; H-2′b), 3.68 (s, 3H; CO_2CH_3), 4.06 (d, $^2J=11.2$ Hz, 1H; H-5′a), 4.18 (d, $^2J=11.2$ Hz, 1H; H-5′b), 4.69 (dd, $^3J=6.5$ Hz, $^3J=2.8$ Hz, 1H; H-3′), 6.60 (dd, $^3J=7.8$ Hz, $^3J=6.0$ Hz, 1H; H-1′), 7.34–7.70 (m, 11H; Ar, H-6), 8.33 ppm (br s, 1H; NH); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta=-5.0$ ($SiCH_3$), −4.8 ($SiCH_3$), 12.0 (CH_3), 18.1 ($SiC(CH_3)_3$), 19.7 ($SiC(CH_3)_3$), 25.8 ($SiC(CH_3)_3$), 27.3 ($SiC(CH_3)_3$), 41.5 (C-2′), 52.3 (CO_2CH_3), 65.5 (C-5′), 73.9 (C-3′), 86.0 (C-1′), 92.4 (C-4′), 111.4 (C-5), 128.2, 128.3, 129.0, 130.3, 130.5, 131.1, 132.4, 133.0, 135.5, 135.67, 135.7, 150.2 (C-2), 163.7 (C-4), 170.0 ppm (CO_2CH_3); FAB MS (3-NBA matrix): m/z: 653.2 $[M+H]^+$, 595.1 $[M-t\mathrm{Bu}]^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-(carboxylic acid methyl ester) thymidine (17): Compound **16** (55.2 mg, 0.09 mmol) and a 1M solution of TBAF (200 μL, 0.20 mmol) in anhydrous THF (4 mL) were stirred at room temperature for 1.5 h. The solvent was then evaporated in a vacuum and the residue was subjected to column chromatography (SiO_2, dichloromethane/methanol 9:1) to yield the desired alcohol as a colorless solid (23.8 mg, 0.08 mmol, 93%); $R_f=0.24$ (dichloromethane/methanol 9:1); ^{1}H NMR (400 MHz, CD_3OD): $\delta=1.87$ (d, $^4J=1.2$ Hz, 3H; CH_3-5), 2.28–2.35 (m, 2H; H-2′a, H-2′b), 3.74 (s, 3H; CO_2CH_3), 3.91 (d, $^2J=12.0$ Hz, 1H; H-5′a), 3.96 (d, $^2J=12.0$ Hz, 1H; H-5′b), 4.57 (dd, $^3J=6.3$ Hz, $^3J=5.2$ Hz, 1H; H-3′), 6.48 (dd, $^3J=^3J=6.6$ Hz, 1H; H-1′), 7.73 ppm (d, $^4J=1.2$ Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta=12.6$ (CH_3), 40.7 (C-2′), 52.7 (CO_2CH_3), 64.4 (C-5′), 73.5 (C-3′), 87.3 (C-1′), 93.5 (C-4′), 111.9 (C-5), 138.5 (C-6), 152.7 (C-2), 166.9 (C-4), 172.6 ppm (CO_2CH_3); FAB MS (3-NBA matrix): m/z: 301.1 $[M+H]^+$, 242.3 $[M-CO_2Me+H]^+$. DMTCl (51.6 mg, 0.15 mmol) and a catalytic amount of DMAP were added at room temperature to a stirred solution of the alcohol (22.6 mg, 0.08 mmol) in pyridine (2 mL). After 5 h the reaction was quenched by the addition of methanol (1 mL) and stirring was continued for 30 min. The solvent was then removed under reduced pressure and the resulting residue was separated by flash column chromatography (SiO_2, cyclohexane/ethyl acetate 1:9+1% triethylamine–ethyl acetate+1% triethylamine). Nucleoside **17** was isolated as a yellowish foam (26.9 mg, 0.05 mmol, 60%); $R_f=0.45$ (cyclohexane/ethyl acetate 1:9); ^{1}H NMR (400 MHz, CD_3OD): $\delta=1.52$ (d, $^4J=1.2$ Hz, 3H; CH_3-5), 2.44–2.52 (m, 2H; H-2′a, H-2′b), 3.60 (d, $^2J=10.0$ Hz, 1H; H-5′a), 3.70 (d, $^2J=10.0$ Hz, 1H; H-5′b), 3.77 (s, 3H; CO_2CH_3), 3.84 (s, 6H; OCH_3), 4.73 (dd, $^3J=6.4$ Hz, $^3J=4.8$ Hz, 1H; H-3′), 6.62 (dd, $^3J=^3J=6.8$ Hz, 1H; H-1′), 6.90–7.50 (m, 13H; Ar), 7.61 ppm (d, $^4J=1.2$ Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta=12.4$ (CH_3), 40.9 (C-2′), 52.7 (CO_2CH_3), 55.9 (OCH_3), 66.6 (C-5′), 74.1 (C-3′), 87.2 (C-1′), 88.3 (CAr_3), 93.0 (C-4′), 112.1 (C-5), 114.36, 114.37, 125.3, 128.3, 129.1, 129.6, 131.57, 131.6, 136.8, 136.9, 137.2, 137.9, 146.1, 149.4, 149.6 (Ar, C-6), 152.8 (C-2), 153.0, 160.52, 160.53 (Ar), 167.1 (C-4), 172.3 ppm (CO_2CH_3); FAB MS (3-NBA matrix): m/z: 602.3 $[M^+]$, 303.1 $[\mathrm{DMT}^+]$.

4-*N*-Benzoyl-3′-*O*-*tert*-butyldimethylsilyl-5′-*O*-*tert*-butyldiphenylsilyl-4′-*C*-methoxymethyl-5-methyl cytidine (23): 2,4,6-Triisopropylbenzenesulfonyl chloride (TPSCl, 95 mg, 0.31 mmol) was added to a solution of **17** (100 mg, 0.16 mmol), DMAP (38.5 mg, 0.32 mmol), and triethylamine (44 μL, 0.32 mmol) in acetonitrile (1 mL). The mixture was stirred for 1.5 h at 0°C, then a solution of 33% NH_4OH/CH_3CN (1 mL, 1:1) was added and stirring was continued at 0°C for 1.5 h. The reaction mixture was allowed to warm up to room temperature and stirring was continued for another 1.5 h. The mixture was then diluted with dichloromethane and poured onto aqueous $KHSO_4$ solution (50 mL, pH 5). The aqueous layer was extracted with CH_2Cl_2, the combined organic phase was dried over $MgSO_4$, and concentrated. The desired cytidine derivative was obtained following purification by flash column chromatography (SiO_2, ethyl acetate–ethyl acetate/methanol 9:1) as a colorless foam (71 mg, 0.11 mmol, 71%); $R_f=0.30$ (ethyl acetate/cyclohexane 1:9); ^{1}H NMR (400 MHz, CD_3OD): $\delta=0.16$ (s, 3H; $SiCH_3$), 0.19 (s, 3H; $SiCH_3$), 1.00 (s, 9H; $SiC(CH_3)_3$), 1.16 (s, 9H; $SiC(CH_3)_3$), 1.69 (d, $^4J=1.1$ Hz, 3H; CH_3-5), 2.25 (ddd, $^2J=13.3$ Hz, $^3J=7.7$ Hz, $^3J=6.0$ Hz, 1H; H-2′a), 2.50 (ddd, $^2J=13.3$ Hz, $^3J=5.9$ Hz, $^3J=2.9$ Hz, 1H; H-2′b), 3.38 (s, 3H; OCH_3), 3.61 (d, $^2J=9.9$ Hz, 1H; H-5′a), 3.65 (d, $^2J=9.9$ Hz, 1H; H-5′b), 3.92 (d, $^2J=11.0$ Hz, 1H; 4′-C-CH_2a), 3.95 (d, $^2J=11.0$ Hz, 1H; 4′-C-CH_2b), 4.65 (dd, $^3J=6.0$ Hz, $^3J=2.9$ Hz, 1H; H-3′), 6.37 (dd, $^3J=7.7$ Hz, $^3J=5.9$ Hz, 1H; H-1′), 7.45–7.77 (m, 10H; Ar), 7.63 ppm (d, $^4J=1.0$ Hz, 1H; H-6); ^{13}C NMR (100.6 MHz, CD_3OD): $\delta=-4.8$ ($SiCH_3$), −4.4 ($SiCH_3$), 13.4 (CH_3), 19.1 ($SiC(CH_3)_3$), 20.4 ($SiC(CH_3)_3$), 26.4 ($SiC(CH_3)_3$), 27.7 ($SiC(CH_3)_3$), 43.2 (C-2′), 59.8 (OCH_3), 66.7 (C-5′), 73.6 (4′-C-CH_2), 74.7 (C-3′), 87.2 (C-1′), 90.6 (C-4′), 104.4 (C-5), 129.16, 129.17, 131.35, 131.4, 134.1, 134.5, 136.8, 139.3 (C-6), 158.3 (C-2), 167.4 ppm (C-4); FAB MS (3-NBA matrix): m/z: 1275.8 $[M+H]^+$, 638.4 $[M+H]^+$; HRMS (ESI): calcd for $C_{34}H_{50}N_3O_5Si_2$: 636.3289; found: m/z: 636.3325 $[M-H]^-$. The cytidine analogue (61.6 mg, 0.1 mmol), benzoic anhydride (44.9 mg, 0.2 mmol), and a catalytic amount of DMAP were dissolved in anhydrous pyridine (1 mL). The solution was stirred under argon for 8 h at room temperature. The solvent was then evaporated and the residue was purified by column chromatography (SiO_2, cyclohexane/ethyl acetate 9:1) to yield 63.3 mg (0.09 mmol, 88%) of a colorless foam (**23**); $R_f=0.30$ (ethyl acetate/cyclohexane 1:9); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.03$ (s, 3H; $SiCH_3$), 0.07 (s, 3H; $SiCH_3$), 0.90 (s, 9H; $SiC(CH_3)_3$), 1.09 (s, 9H; $SiC(CH_3)_3$), 1.79 (d, $^4J=1.0$ Hz, 3H; CH_3-5), 2.19 (ddd, $^2J=13.1$ Hz, $^3J=8.3$ Hz, $^3J=5.8$ Hz, 1H; H-2′a), 2.36 (ddd, $^2J=13.1$ Hz, $^3J=5.6$ Hz, $^3J=2.0$ Hz, 1H; H-2′b), 3.30 (s, 3H; OCH_3), 3.44 (d, $^2J=10.2$ Hz, 1H; H-5′a), 3.54 (d, $^2J=10.2$ Hz, 1H; H-5′b), 3.82 (d, $^2J=11.0$ Hz, 1H; 4′-C-CH_2a), 3.90 (d, $^2J=11.0$ Hz, 1H; 4′-C-CH_2b), 4.55 (dd, $^3J=5.8$ Hz, $^3J=2.0$ Hz, 1H; H-3′), 6.38 (dd, $^3J=8.3$ Hz, $^3J=5.6$ Hz, 1H; H-1′), 7.36–7.68 (m, 16H; Ar, H-6), 8.27 (d, $^4J=1.4$ Hz, 1H; Ar), 8.29 ppm (d, $^4J=1.4$ Hz, 1H; Ar); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta=-5.0$ ($SiCH_3$), −4.5 ($SiCH_3$), 13.4 (CH_3), 18.3 ($SiC(CH_3)_3$), 19.6 ($SiC(CH_3)_3$), 25.9 ($SiC(CH_3)_3$), 27.2 ($SiC(CH_3)_3$), 42.3 (C-2′), 59.7 (OCH_3), 65.9 (C-5′), 72.9 (4′-C-CH_2), 73.5 (C-3′), 85.3 (C-1′), 89.7 (C-4′), 111.9 (C-5), 128.18, 128.2, 128.3, 130.0, 130.3, 130.4, 132.6, 132.7, 133.2, 135.6, 135.8, 136.9, 137.5 (Ar, C-6), 148.1 (C-2), 160.0 (C-4), 179.8 ppm ($COCH_2Ph$); FAB MS (3-NBA matrix): m/z: 742.3 $[M+H]^+$; HRMS (ESI): calcd for $C_{41}H_{55}N_3O_6Si_2$: 740.3551; found: m/z: 740.3566 $[M-H]^-$.

4-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-4′-*C*-methoxymethyl-5-methyl cytidine (24): A 1M solution of TBAF (0.17 mL, 0.17 mmol) was added to a solution of **23** (58.8 mg, 0.08 mmol) in 1 mL anhydrous THF and the

preparation was stirred at room temperature for 4 h. The solvent was then removed in a vacuum and the residue was subjected to column chromatography (SiO_2, ethyl acetate/methanol 9:1) to isolate the desired intermediate, which was used in the next reaction step. The residue was coevaporated and redissolved in anhydrous pyridine (1 mL). DMTCl (76.4 mg, 0.23 mmol) and a catalytic amount of DMAP were added to the resulting solution at 0 °C. After being stirred for 30 min, the mixture was allowed to warm up to room temperature and stirring was continued for 5 h. The reaction was then quenched by the addition of methanol (4 mL) and after 30 min evaporated to dryness. Purification of the resultant residue by flash column chromatography (SiO_2, cyclohexane/ethyl acetate 2:3+1 % triethylamine) furnished 38.5 mg (0.06 mmol, 71 %) of a faint yellowish foam (**24**); R_f=0.45 (ethyl acetate/cyclohexane 7:3); ^{1}H NMR (400 MHz, CD_3OD): δ=1.72 (d, ^4J=1.0 Hz, 3H; CH_3-5), 2.34 (m, 1H; H-2′a), 2.60 (m, 1H; H-2′b), 3.36 (d, ^2J=10.0 Hz, 1H; H-5′a), 3.41 (s, 3H; OCH_3), 3.47 (d, ^2J=10.0 Hz, 1H; H-5′b), 3.65 (d, ^2J=9.9 Hz, 1H; 4′-C-CH_2a,), 3.69 (d, ^2J=9.9 Hz, 1H; 4′-C-CH_2b), 3.83 (s, 3H; OCH_3), 3.84 (s, 3H; OCH_3), 4.67 (dd, ^3J=6.8 Hz, ^3J=4.4 Hz, 1H; H-3′), 6.20 (dd, ^3J=^3J=6.5 Hz, 1H; H-1′), 6.90–7.92 (m, 17H; Ar, H-6), 8.08 (dd, ^3J=8.5 Hz, ^4J=1.3 Hz, 1H; Ar), 8.20 ppm (d, ^3J=7.1 Hz, 1H; Ar); ^{13}C NMR (100.6 MHz, CD_3OD): δ=13.2 (CH_3), 42.8 (C-2′), 55.9 (OCH_3), 59.9 (OCH_3), 66.2 (C-5′), 73.4 (4′-C-CH_2), 74.2 (C-3′), 87.1 (C-1′), 88.3 (C-4′), 89.9 (CAr_3), 104.3 (C-5), 114.4, 128.2, 129.0, 129.1, 129.6, 129.63, 129.7, 130.6, 131.5, 131.58, 131.6, 134.3, 136.9, 137.0, 137.1, 137.13, 139.7, 146.2 (Ar, C-6), 158.3 (C-2), 160.5 (C-4), 167.4 ppm ($COCH_2Ph$); FAB MS (3-NBA matrix): m/z: 588.3 $[M-Bz+2H]^+$, 303.1 $[DMT^+]$; HRMS (ESI): calcd for $C_{40}H_{40}N_3O_8$: 690.2815; found: m/z: 690.2835 $[M-H]^-$.

General procedure for coupling of 5′-*O*-protected nucleosides to succinylated LCAA-CPG: Compounds **3a–c**, **13**, **17**, and **24** were coupled to succinylated LCAA-CPG by using standard protocols.[46] Briefly, succinylated LCAA-CPG, the respective nucleosides **3a–c**, **13**, **17**, or **24**, DMAP (each 0.1 mmol/1.0 g CPG), and EDC (1.0 mmol/1.0 g CPG), were combined, pyridine (10 mL/1.0 g CPG) and NEt_3 (80 μL/1.0 g CPG) were added, and the reaction mixture was shaken under argon overnight. Next, 4-nitrophenol (0.5 mmol/1.0 g CPG) was added and shaking was continued for an additional 24 h. Piperidine (5 mL/1.0 g CPG) was added, and shaking was continued for 5 min. The beads were then filtered off and washed successively with pyridine, methanol, and finally with CH_2Cl_2. After drying, the beads were suspended in equal amounts of acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B) capping reagents. After shaking for 2 h, the beads were filtered off and intensively washed as described above. After drying, loading was determined by trityl analysis of a small portion of the collected beads (loading range 8.5–31.9 μmol g^{-1}).[46]

Synthesis of 4′-*C*-modified oligonucleotides: The synthesis of oligonucleotides was performed on a 0.2 μmol scale by using an Applied Biosystems 392 DNA synthesizer and commercially available 2-(cyanoethyl)-phosphoramidites. A standard method for the synthesis of 2-(cyanoethyl) phosphoramidites was used, with the exception that the coupling times for the modified nucleotides were extended to 10 min. Yields for modified oligonucleotides were similar to those obtained for unmodified oligonucleotides. After synthesis (trityl off) the oligonucleotides were cleaved from the support by treatment with 33 % NH_4OH at 55 °C for 12 h. After removal of NH_4OH the residue was purified by preparative electrophoresis through a 12 % polyacrylamide gel containing 8 M urea. For the synthesis of primer probes **Far7** and **Far9**, **18** was used for oligonucleotide synthesis and subsequently treated with 0.5 M NaOH or 2 M NaOMe (MeOH/H_2O 4:1), respectively, for 22 h at room temperature, then neutralized with 2 M triethylammonium acetate (TEAA), and desalted (NAP-25 column, Amersham Biosciences). After evaporation the residues were purified by preparative PAGE, as described above. The DNA oligonucleotides were recovered by standard precipitation with ethanol in the presence of 0.3 M sodium acetate. The oligonucleotides were quantified by measuring absorption at 260 nm. Total yields of purified oligonucleotides were in the range of 25–33 %. The integrity of all modified oligonucleotides was confirmed by performing MALDI-ToF or ESI-FTICR MS.

Real-time PCR experiments: Real-time PCR was performed by using an iCycler System (Bio-Rad). In brief, the reactions were performed in a total volume of 50 μL, which contained 4 pmol of the respective templates in the respective buffers provided by the supplier for Vent (exo−) DNA polymerase (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X-100). The final mixtures contained dNTPs (200 μM each of dATP, dGTP, dCTP, and TTP), primers (0.5 μM each of respective primer probe and reverse primer), 1.2 units of Vent (exo−) DNA polymerase (units defined by the supplier, New England Biolabs), and a 1:25000 aqueous dilution of a 10000x solution of *SybrGreen I* in DMSO (Molecular Probes). All PCR amplifications were performed by employing the following program: initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 35 s, and extension at 72 °C for 40 s. The data presented were obtained from independent measurements of triplicates originating from one master-mix. All experiments were repeated at least two times. To enable comparison with previous studies, the DNA sequences of template target, primer probe, and reverse primer were identical to those employed previously. Sequences in the Farber disease context: Primer probe 5′-d(CGT TGG TCC TGA AGG AGG A**N^R**), reverse primer: 5′-d(CGC GCA GCA CGC GCC GCC GT), target template FarX: 5′-d(CCG TCA GCT GTG CCG TCG CGC AGC ACG CGC CGC CGT GGA CAG AGG ACT GCA GAA AAT CAA CCT **X**TC CTC CTT CAG GAC CAA CGT ACA GAG); **X**: A, FarA; G, FarG. Sequences in the Factor V Leiden disease context: Primer probe 5′-d(CAA GGA CAA AAT ACC TGT ATT CCT **N^R**), reverse primer: 5′-d(GAC ATC ATG AGA GAC ATC GC), target template LeiX: 5′-d(GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG C**X**A GGA ATA CAG GTA TTT TGT CCT TG); **X**: A, LeiA; G, LeiG. Sequences in the DPyD context: Primer probe DpyDT^R: 5′-d(GTT TTA GAT GTT AAA TCA CAC TTA **N^R**), reverse primer: 5′-d(AAA GCT CCT TTC TGA ATA TTG AG), target template DPyDX: 5′-d(AAA ATG TGA GAA GGG ACC TCA TAA AAT ATG TCA TAT GGA AAT GAG CAG ATA ATA AAG ATT ATA GCT TTT CTT TGT CAA AAG GAG ACT CAA TAT CTT TAC TCT TTC ATC AGG ACA TTG TGA CAA ATG TTT CCC CCA GAA TCA TCC GGG GAA CCA CCT CTG GCC CCA TGT ATG GCC CTG GAC AAA GCT CCT TTC TGA ATA TTG AGC TCA TCA GTG AGA AAA CGG CTG CAT ATT GGT GTC AAA GTG TCA CTG AAC TAA AGG CTG ACT TTC CAG ACA AC**X** TAA GTG TGA TTT AAC ATC TAA AAC); **X**: A, DPyDA; G, DPyDG.

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