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Research on L-Nucleosides. Synthesis and Biological Evaluation of a Series of L- and D-2′,3′-Dideoxy-3′-[tris(methylthio)methyl]-β-pentofuranosyl Nucleosides

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Abstract—Novel nucleoside analogues of both D and L enantiomeric series were prepared by coupling reaction between a 2′,3′-dideoxy-3′-modified furanose moiety and four different nucleobases. Though in all cases anomeric mixtures of nucleosides were obtained, the presence of the sterically bulky 3′-tris(methylthio)methyl group allowed a good stereoselectivity level. All the compounds of both enantiomeric series showed high IC_{50} values as HSV-1 TK inhibitors and scarce ability to be phosphorylated by HSV-1 TK. In order to overcome possible problems related to the first phosphorylation step and to facilitate the penetration of the molecule through the cellular membrane, a monophosphate prodrug containing a long lipophilic chain was synthesized. No appreciable antiviral activity was exhibited by this molecule.

Introduction

Nucleoside analogues have been the cornerstone of antiviral therapy over the past 30 years[1] and a variety of strategies have been exploited to design new nucleoside analogues able to block viral replication without affecting host cellular processes.[2] However, the toxicities associated with certain nucleoside analogues and the emergence of resistant viral strains warrant the search for further novel and structurally diverse compounds with minimally overlapping resistance profile and toxicity.[3]

It was long supposed that only nucleosides with the natural β-D-configuration at the 4′-position would be substrates for enzymatic phosphorylation and hence able to act as antiviral agents. As a result, only sporadic examples of β-L-nucleosides were reported in the literature until the early 1990s, when certain L-nucleosides like 3TC (L-2′,3′-dideoxy-3′-thiacytidine, Lamivudine, **1**) and FTC (L-2′,3′-dideoxy-3′-thia-5-fluorocytidine, **2**) (Fig. 1) proved to possess more potent antiviral activity against human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) compared to their D-counterparts while exhibiting less toxicity.[4] Although only one L-nucleoside (Lamivudine) has so far gained approval by FDA for the treatment of AIDS, other

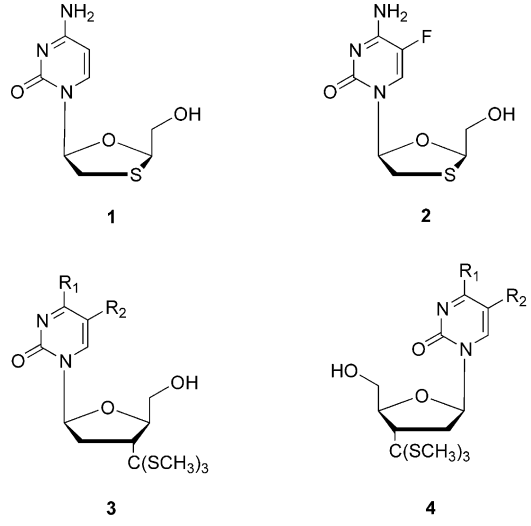

Figure 1.

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L-enantiomers of nucleoside analogues are currently under development as antiviral or antitumor agents.[5] Therefore, it is of interest to investigate new L-nucleosides in search of antiviral agents with favorable toxicity profile.[6]

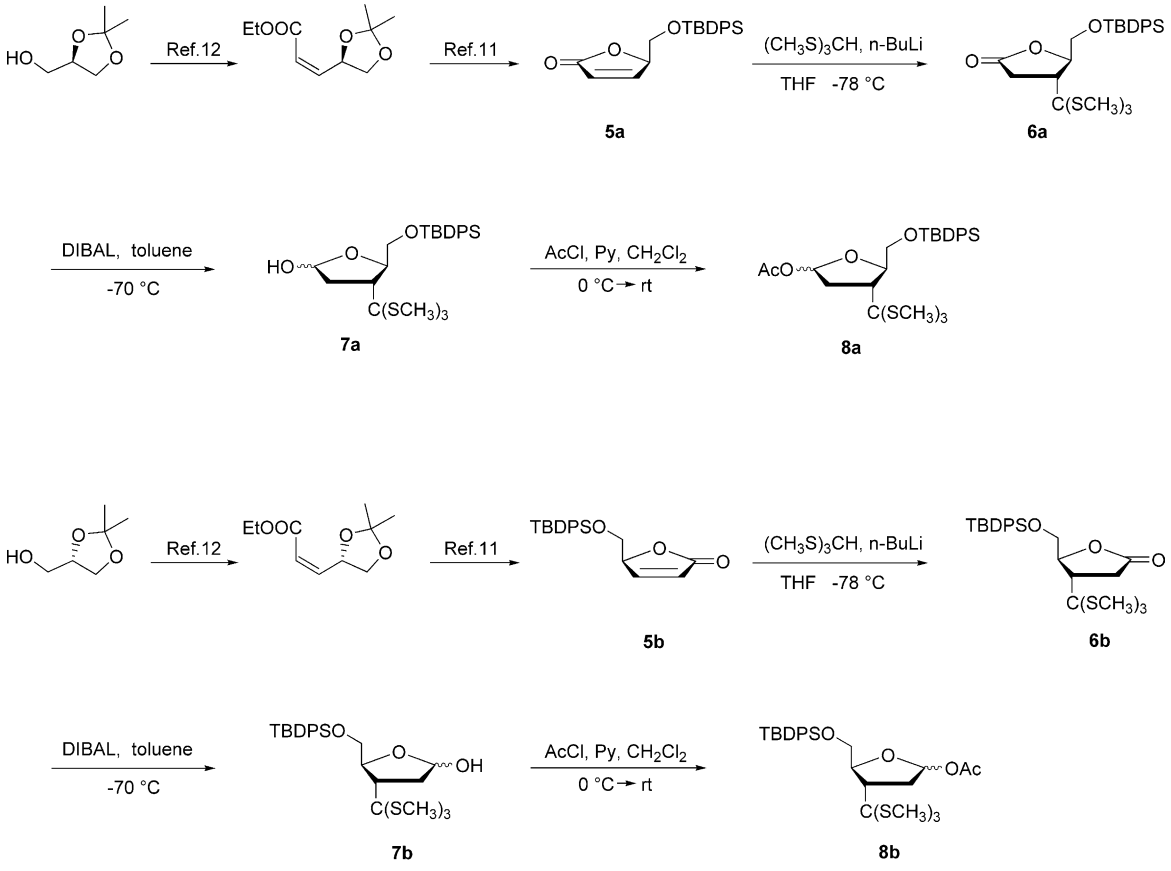
As part of our efforts to develop new antiviral agents,[7] we decided to synthesize a series of L-nucleosides of general structure **3**, characterized by the presence of a tris(methylthio)methyl group at the 3′-position of the sugar moiety, in order to test them as potential antiviral agents against herpes simplex virus (HSV) in comparison with their D-enantiomers **4**. Several considerations prompted us to select this type of compounds as synthetic target:

i. the modification of the pentofuranose ring is one of the most effective among all the strategies devised to design new nucleoside analogues
ii. compounds **3** and **4** can be considered as 2′,3′-dideoxynucleosides just like the majority of the nucleoside antivirals approved so far
iii. introduction of electronwithdrawing substituents, such as azido and fluorine, at the 3′-position has often enhanced the antiviral potency[8]
iv. the presence of a bulky group at the 3′-position can improve the β/α anomeric ratio during the coupling reaction between pyrimidine bases and the sugar moiety
v. the tris(methylthio)methyl group is chemically versatile and can be transformed into different functionalities.

Results and Discussion

Chemistry

For the synthesis of the target nucleoside analogues we adopted a convergent approach[9] based on the coupling reaction between an appropriately activated sugar moiety and a silylated pyrimidine base, according to the Vorbrüggen conditions.[10] The α,β-unsaturated lactones **5a** (L-enantiomer) and **5b** (D-enantiomer) (Scheme 1) were prepared following known procedures[11] and exploiting an improved one-pot methodology for the direct conversion of (*R*)- and (*S*)-2,3-*O*-isopropylideneglycerol into ethyl (*Z*)-3-[(4*R*)-2,2-dimethyl-1,3-dioxolan-4-yl]-2-propenoate and the corresponding (4*S*)-enantiomer, respectively.[12] Starting from **5a**, compound **6a** was prepared in 81% yield using the same conditions reported by Hanessian et al.[13] for the synthesis of **6b** from **5b**. Reduction with DIBAL of the lactones **6a**,**b** to lactols **7a**,**b**, followed by acetylation under standard conditions, led to the 3′-substituted sugar derivatives **8a** and **8b**, suitably protected and activated for the coupling step, in 62% overall yield.



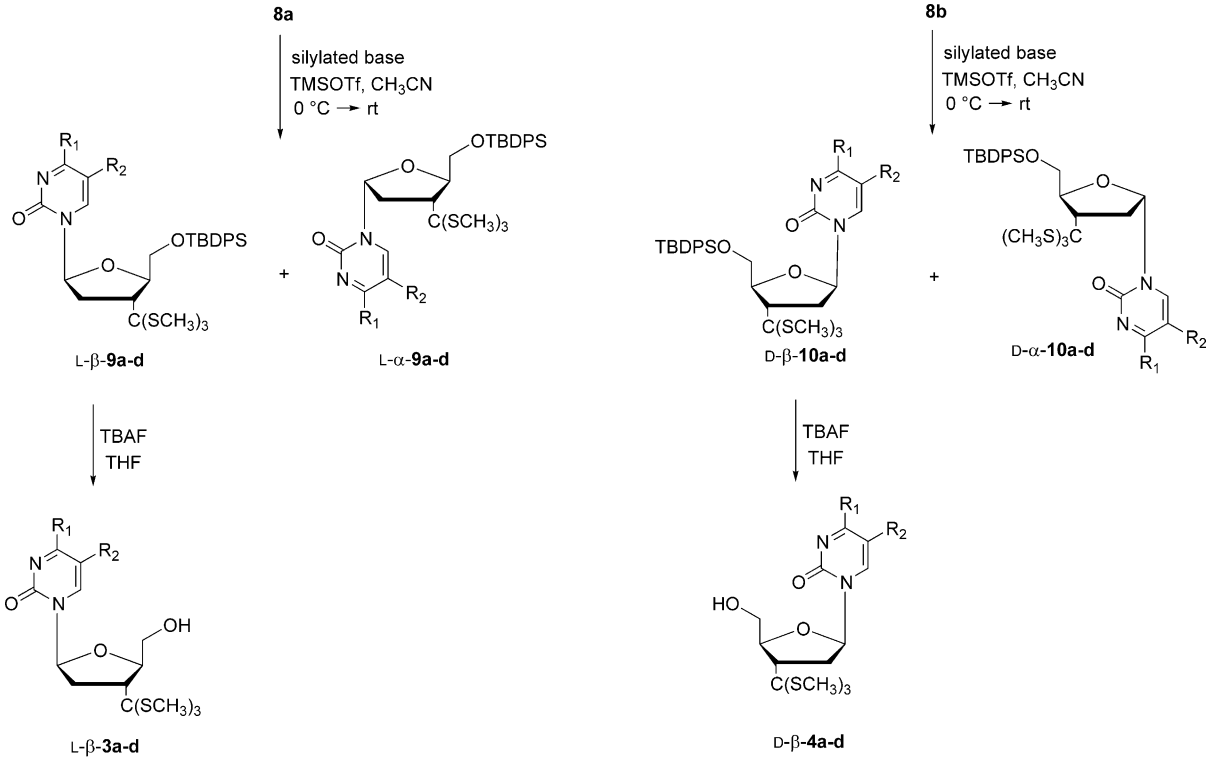
Scheme 1.

The synthesis of 2′-deoxynucleosides represents a rather difficult task in nucleoside chemistry, since derivatives of 2′-deoxysugars are often unstable and their nucleosidation reaction frequently suffers from low total yield and scarce stereoselectivity, that leads to unfavorable ratios between the natural β-anomer and the undesired α-epimer.[10] For the preparation of the target nucleoside analogues **3** and **4**, the Vorbrüggen approach was followed to couple the activated sugar **8a** and **8b** with persilylated uracil, 5-fluorouracil, thymine, and cytosine, using trimethylsilyl trifluoromethanesulfonate (TMSOTf) as a catalyst in acetonitrile (Scheme 2). All of the nucleobases employed underwent *N*-glycosylation reaction to give protected nucleosides **9a–d** and **10a–d** as anomeric mixtures, which could be separated into individual anomers by chromatography. In all cases the overall yield of the coupling reaction was good, ranging from 55 to 77%, and comparable to those reported in the literature for other 2′-deoxynucleoside analogues, while the β/α ratios (4/1 to 11/1) were far higher than those usually obtained for 2′-unsubstituted nucleosides (β/α ratio≈1/1),[8,14] thus definitely demonstrating the positive influence of the bulky *erythro*-3′-substituent on the stereochemical outcome of the reaction.

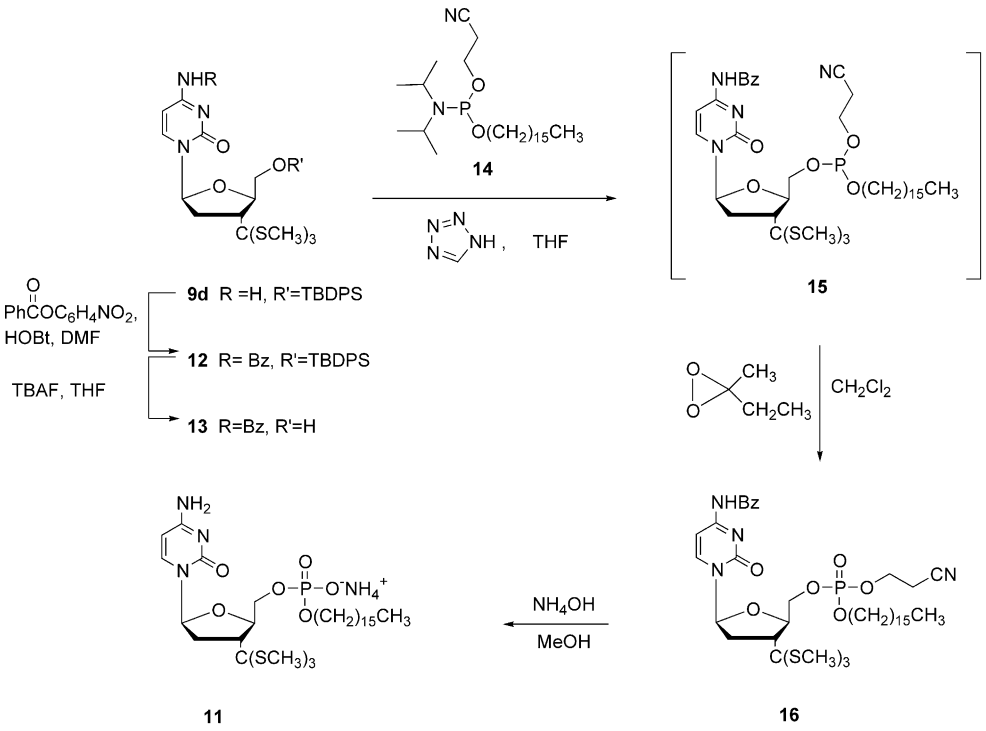
The structure of α- and β-anomers was assigned on the basis of their proton NMR spectra, according to the method previously used by Okabe et al.[14] for D-nucleosides and by Lin et al.[15] for L-nucleosides. The H-4′ proton of the α-anomer appears at a lower field than that of the β-anomer, while the H-5′ protons of the α-anomers appear at a higher field than those of the β-anomers. Contrary to previous findings,[16] no relation was found between anomers stereochemistry and polarity.

After chromatographic separation of the anomers, deprotection of L-β-**9a–d** and D-β-**10a–d** with TBAF in THF afforded the final nucleoside analogues **3a–d** and **4a–d** in 90–95% yield. The corresponding α-anomers were not further considered and hence not subjected to the deprotection step.

As a consequence of the biological testing of these compounds (see below), the question arose whether their modest antiviral activity might be due to a low rate and/or extent of conversion to their active (triphosphate) forms by cellular enzymes rather than to the scarce affinity of the triphosphates for the target enzyme. Since one way to improve the efficiency of nucleosides should be to bypass at least in part these phosphorylation steps, we decided to prepare the monophosphate prodrug **11** (Scheme 3) in which the phosphate is modified by a hydrophobic moiety in order to enhance its cell penetration capability.[17] As the nucleoside substrate to be subjected to this strategy we chose compound **3d** because it is easily available as the β-anomer and shows an interesting antiviral profile. To this end **9d** was *N*-benzoylated using 4-nitrophenyl benzoate to give **12** which was selectively deprotected to give **13**. According to the method of nucleoside phosphorylation based on phosphoramidite reagents,[18] 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphoramidite **14**[17,19] was prepared and used without purification in the next step. Reaction of **14** with the *N*-protected nucleoside **13** in the presence of tetrazole gave the phosphite triester **15**, which was not isolated but directly oxidized to the corresponding phosphate **16**. Although different reagents have been reported in the literature to accomplish this oxidation step,[17,20] the remarkable



Scheme 2. a: R_1 = OH, R_2 = H; b: R_1 = OH, R_2 = F; c: R_1 = OH, R_2 = Me; d: R_1 = NH_2, R_2 = H.



Scheme 3.

reactivity of the tris(methylthio)methyl group toward oxidizing agents and thiophilic promoters suggested the use of a reagent able to selectively oxidize the phosphite moiety. According to a very recent literature report,[21] ethyl(methyl)dioxirane perfectly met this requirement and allowed to obtain phosphate **16** in 23% overall yield. Treatment of **16** with concentrated aqueous ammonia overnight, followed by TLC purification, afforded the hexadecyl phosphate **11** (ammonium salt) in 20% yield.

Biological studies

The remarkable anti-HSV activity of several nucleoside analogues, such as Acyclovir and Gancyclovir, is ultimately due to a specific interaction of their 5′-triphosphates with the viral DNA polymerase, but relies on their prior phosphorylation to nucleoside mono- or diphosphates by the virally encoded thymidine kinase (TK).[5c] The HSV TKs differ from those of animal cells both in their greater affinity for the substrate thymidine (and deoxycytidine) and their wide acceptance of nucleosides modified either in the pyrimidine ring or in the sugar moiety as alternative substrates.[22] The ability of purified HSV TKs to recognize and phosphorylate in vitro the L-enantiomer of the naturally occurring substrate D-β-deoxythymidine has been investigated by some of us.[23] On the other hand, Balzarini et al. studied the interaction of the same enzyme with the D- and L-enantiomers of the carbocyclic analogues of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine and 5-iodo-2′-deoxyuridine.[24] Both groups demonstrated that HSV TK, contrary to human TK, is not enantioselective, being able to phosphorylate with similar efficiency both D- and L-enantiomers of the tested nucleosides.

Based on these findings, the synthesized compounds L-β-**9a–d** and D-β-**10a–d** were evaluated in vitro for their cytotoxicity against normal lymphocytes and different tumor lines [NSO cells (plasmocytoma murine cell line), 3T3 cells (murine fibroblast line), and Daudi cells (human lymphoblastoid cell line)] as well as against HSV-1 TK and the results are shown in Table 1. All of the tested compounds proved to be not cytotoxic against normal and tumor cell lines, with ED_{50} values approximately in the range 100–400 μM. On the other hand, none of the synthesized nucleoside analogues exhibited any ability to act as substrates or inhibitors of human thymidine phosphorylase (data not shown), a potential target for tumor-dependent angiogenesis.[25] As a result, these compounds are not promising as potential antitumor agents.

As far as the antiviral activity is concerned, in most cases L-enantiomers were found to be more potent

Table 1. Biological evaluation of compounds **3–4**

Compd	Cytotoxicity assay		Anti-HSV-1 TK assay
	NL[a] ED_{50} (μM)	NSO[b] ED_{50} (μM)	IC_{50} (μM)
3a	332	398	No inhibition
3b	207	230	35
3c	76	105	227
3d	314	383	45
4a	240	330	No inhibition
4b	205	77	167
4c	207	275	No inhibition
4d	234	320	No inhibition

[a]NL, normal lymphocytes.
[b]NSO, plasmocytoma murine cell line.

HSV-1 TK inhibitors than the corresponding D-counterparts: in particular L-β-**3b** and D-β-**4b**, having 5-fluorouracil as the nucleobase, emerged as the most interesting compound within both enantiomeric series. However, when tested as possible substrates of viral TK, following the in vitro assay and HPLC analysis described in Experimental, these nucleoside analogues were found resistant to the phosphorylation activity of the enzyme. In fact, in assay conditions which allow the phosphorylation of 1.2 nmol of the natural substrate thymidine, we did not observe any significant phosphorylation of the tested nucleoside analogues. Therefore, because of their high IC_{50} values (35 and 167 μM, respectively) and their inability to be phosphorylated by HSV-1 TK, we did not perform further biological evaluation.[26] In conclusion, the compounds which show IC_{50} values against HSV-1 TK can be only considered non-substrate inhibitors of the enzyme. To our disappointment, neither phosphate prodrug **11** nor the fully protected triester **16** exhibited any appreciable antiviral activity: they did not reduce either the incorporation of exogenous [^{3}H]thymidine into cellular DNA in HeLa TK^-/HSV-1 TK^+ or HSV-1 multiplication in HeLa cells following the methods described by Salvetti et al.[22]

Conclusion

In summary, a novel class of L- and D-configuration nucleoside analogues has been synthesized, which possess a modified sugar moiety characterized by a bulky tris(methylthio)methyl group at the 3′-position. The steric hindrance exerted by this group caused the nucleosidation reaction to occur with a much higher stereoselectivity than that usually observed for other 2′-deoxynucleosides. Although the biological activity of these compounds is not exciting, it might be possible to obtain new derivatives endowed with better antiviral and/or antitumor properties by modifying the chemically reactive tris(methylthio)methyl group. Studies in this direction are in progress.

Experimental

Chemistry

Unless otherwise stated, all reactions were carried out under an argon atmosphere. Reagents were obtained from commercial suppliers and used without further purifications. Merck silica gel 60 was used for both column chromatogaphy (70–230 mesh) and flash chromatography (230–400 mesh). Melting points were determined in open capillary tubes on a Gallenkamp apparatus and are uncorrected. ^{1}H NMR spectra were measured at 200 MHz with a Bruker AC200F spectrometer. Chemical shifts are reported relative to $CDCl_3$ at δ 7.24 ppm and tetramethylsilane at δ 0.00 ppm. Infrared spectra were recorded on a Perkin–Elmer 1600 FT-IR spectrophotometer. EI and FAB low-resolution mass spectra were recorded with an electron beam of 70 eV using a VG 70–250S instrument. Specific rotations were measured on a Perkin–Elmer Model 343 polarimeter. Elemental analyses (C, H, N) were performed in house using a Perkin–Elmer Elemental Analyzer 240C.

(4*R*,5*R*)-5-({[1-(*tert*-Butyl)-1,1-diphenylsilyl]oxy}methyl)-4-[tri(methylthio)methyl]tetrahydro-furan-2-one (6a). To a solution of tris(methylthio)methane (1.13 mL, 8.5 mmol) in anhydrous THF (100 mL) kept at −78 °C *n*BuLi (6.4 mL of a 1.6 M solution in hexane, 10.2 mmol) was added over 3–4 min. After 30 min a solution of **5a** (3.0 g, 8.5 mmol) in THF (20 mL) was added dropwise and the reaction was stirred at −78 °C for 1 h. The mixture was then poured into Et_2O (250 mL) and washed with a saturated solution of NH_4Cl (300 mL). The aqueous phase was extracted with Et_2O (3×200 mL) and the combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. Filtration and removal of the solvent gave 4.5 g of a white solid. Purification of the crude mixture by column chromatography (petroleum ether/Et_2O, 3/1) afforded **6a** (3.5 g, 81%) as a white solid: mp 140–142 °C; $[\alpha]_D^{20} = -18.2$ (*c* 1.10, $CHCl_3$). IR ($CHCl_3$): 1785, 1185, 1120 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.67–7.63 (m, 4H), 7.47–7.37 (m, 6H), 4.84 (ddd, *J* = 2.7, 2.3, 2.3 Hz, 1H), 3.95 (dd, *J* = 11.4, 2.7 Hz, 1H), 3.62 (dd, *J* = 11.4, 2.3 Hz, 1H), 2.99 (ddd, *J* = 10.1, 2.8, 2.3 Hz, 1H), 2.95 (dd, *J* = 18.3, 2.8 Hz, 1H), 2.83 (dd, *J* = 18.3, 10.1 Hz, 1H), 2.11 (s, 9H), 1.04 (s, 9H). Anal. calcd for $C_{25}H_{34}O_3S_3Si$: C, 59.25; H, 6.76. Found: C, 59.41; H, 6.74.

(4*R*,5*R*)-5-({[1-(*tert*-Butyl)-1,1-diphenylsilyl]oxy}methyl)-4-[tri(methylthio)methyl]tetrahydro-furan-2-ol (7a). To a solution of **6a** (1.35 g, 3.0 mmol) in anhydrous toluene (37 mL), maintained at −70 °C, a 1.0 M solution of DIBAL in hexane (7.1 mL, 7.12 mmol) was added dropwise during 5 min. The mixture was stirred for 1 h at −70 °C, then MeOH (50 mL) was added, and the mixture allowed to warm to room temperature. The resulting white precipitate was filtered off and washed with MeOH. The filtrates were combined and evaporated to give a pale yellow oil (1.3 g, 96%) which was used without any further purification: $[\alpha]_D^{20} = -12.5$ (*c* 4.40, $CHCl_3$). IR ($CHCl_3$): 3409, 3007, 2951, 2862, 1731, 1424 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.72–7.64 (m, 4H), 7.44–7.36 (m, 6H), 5.58–5.46 (m, 1H), 4.45–4.40 (m, 1H), 4.08 (dd, *J* = 9.2, 3.0 Hz, 1H), 3.90 (dd, *J* = 2.5, 11.3 Hz, 1H), 3.66 (dd, *J* = 2.9, 10.9 Hz, 1H), 3.12–3.01 (m, 1H), 2.59–2.43 (m, 1H), 2.08 (s, 9H), 1.10 (s, 9H).

(4*S*,5*S*)-5-({[1-(*tert*-Butyl)-1,1-diphenylsilyl]oxy}methyl)-4-[tri(methylthio)methyl]tetrahydrofuran-2-ol (7b). Prepared in 95% yield starting from **6b** using the same procedure described for the synthesis of **7a**: $[\alpha]_D^{20} = +12.1$ (*c* 4.40, $CHCl_3$).

(4*R*,5*R*)-5-({[1-(*tert*-Butyl)-1,1-diphenylsilyl]oxy}methyl)-4-[tri(methylthio)methyl]tetrahydrofuran-2-yl acetate (8a). Lactol **7a** (3 g, 5.9 mmol) was dissolved in anhydrous CH_2Cl_2 (14 mL) and then pyridine (4 mL) was added. Freshly distilled acetyl chloride (1.3 mL, 18.3 mmol) was slowly added to this solution at 0 °C. The resulting white suspension was stirred at room temperature for 1.5 h. The reaction was then quenched with saturated aqueous NH_4Cl solution (5 mL). The mixture

was extracted with CH_2Cl_2 (3×6 mL) and the combined extract was washed with brine and dried over anhydrous Na_2SO_4. Concentration in vacuo gave 4 g of a yellow oil. The crude material was subjected to column chromatography (petroleum ether/AcOEt, 8/1) to provide **8a** (2.1 g, 65%) as a colourless syrup: $[\alpha]_D^{20} = -25.4$ (*c* 3.74, $CHCl_3$). IR ($CHCl_3$): 3682, 3494, 3018, 2860, 1711, 1427 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.71–7.68 (m, 4H), 7.39–7.32 (m, 6H), 6.40–6.31 (m, 1H), 4.50–4.47 (m, 1H), 4.08 (dd, *J* = 2.7, 10.6 Hz, 1H), 3.84 (dd, *J* = 2.7, 11.3 Hz, 1H), 3.27–3.15 (m, 1H), 3.05–2.94 (m, 1H), 2.79–2.65 (m, 1H), 2.16 (s, 9H), 2.14 (s, 9H), 2.06 (s, 3H), 1.82 (s, 3H), 1.07 (s, 9H), 1.05 (s, 9H). Anal. calcd for $C_{27}H_{38}O_4S_3Si$: C, 58.87; H, 6.95. Found: C, 58.69; H, 7.10.

(4*S*,5*S*)-5-({[1-(*tert*-Butyl)-1,1-diphenylsilyl]oxy}methyl)-4-[tri(methylthio)methyl]tetrahydrofuran-2-yl acetate (8b). Prepared in 70% yield from **7b** as described for **8a**: $[\alpha]_D^{20} = +23.9$ (*c* 3.74, $CHCl_3$). Anal. calcd for $C_{27}H_{38}O_4S_3Si$: C, 58.87; H, 6.95. Found: C, 59.12; H, 6.74.

General procedure for the synthesis of nucleosides L–β-9a–d and D-β-10a–d

The appropriate nucleobase (2.2 mmol) was treated with a large excess of HMDS (6 mL) and a catalytic amount of anhydrous $(NH_4)_2SO_4$. The white suspension was refluxed for 2–4 h giving a colourless solution. Removal of HMDS by codistillation under reduced pressure with dry toluene (3×1 mL) gave a white solid residue. The solid was dissolved in dry CH_3CN (6 mL), the solution was cooled to 0 °C and TMSOTf (330 μL, 1.6 mmol) was added dropwise to the mixture. Finally, a solution of **8** (1.1 mmol) in dry CH_3CN (3 mL) was added and the reaction was stirred at room temperature for 45 min. The mixture was then quenched with a saturated aqueous solution of $NaHCO_3$ (10 mL) and extracted with $CHCl_3$ (3×15 mL). The combined organic phase was then washed with brine, dried on anhydrous Na_2SO_4 and filtered. Evaporation of the solvent followed by column chromatography gave the crude nucleoside.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-uracil (L-β-9a) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-L-*erythro*-pentofuranosyl]-uracil (L-α-9a). These compounds were obtained starting from **8a** and uracil in 63 and 11% yield, respectively, after silica gel column chromatography ($CHCl_3$/MeOH, 98/2) followed by further purification by preparative TLC (three runs, $CHCl_3$/MeOH, 95/5).

L-β-9a. White solid, mp 65–67 °C. R_f 0.3. $[\alpha]_D^{20} = -43.8$ (*c* 1.14, $CHCl_3$). IR ($CHCl_3$): 4201, 3025, 1689 cm^{-1}. ^1H NMR ($CDCl_3$): δ 9.18 (br s, 1H), 7.88 (d, *J* = 8.4 Hz, 1H), 7.65–7.59 (m, 4H), 7.41–7.37 (m, 6H), 6.40 (t, *J* = 6.6 Hz, 1H), 5.39 (d, *J* = 8.1 Hz, 1H), 4.45–4.44 (m, 1H), 4.08 (dd, *J* = 1.5, 11.8 Hz 1H), 3.76 (dd, *J* = 2.5, 11.7 Hz, 1H), 3.04–2.87 (m, 3H), 2.10 (s, 9H), 1.08 (s, 9H). MS (FAB) *m/z* 603 [M + H$^+$]. Anal. calcd for $C_{29}H_{38}N_2O_4S_3Si$: C, 57.77; H, 6.35; N, 4.65. Found: C, 57.62; H, 6.49; N, 4.32.

L-α-9a. Colourless oil. R_f 0.2. ^1H NMR ($CDCl_3$) δ 7.75 (d, *J* = 8.2 Hz, 1H), 7.67–7.59 (m, 4H), 7.49–7.33 (m, 6H), 6.35–6.32 (m, 1H), 5.75 (d, *J* = 7.9 Hz, 1H), 4.46–4.45 (m, 1H), 3.92 (dd, *J* = 11.6, 1.7 Hz, 1H), 3.69 (dd, *J* = 11.5, 3.1 Hz, 1H), 3.18–3.11 (m, 1H), 2.64–2.56 (m, 1H), 2.30–2.21(m, 1H), 2.12 (s, 9H), 1.07 (s, 9H). MS (FAB) *m/z* 603 [M + H$^+$]. Anal. calcd for $C_{29}H_{38}N_2O_4S_3Si$: C, 57.77; H, 6.35; N, 4.65. Found: C, 57.58; H, 6.21; N, 4.46.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-uracil (D-β-10a) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-D-*erythro*-pentofuranosyl]-uracil (D-α-10a). These compounds were prepared in 66 and 10% yield, respectively, as described for L-β-9a starting from **8b** and uracil.

D-β-10a. White solid, mp 64–66 °C; $[\alpha]_D^{20} = +44.5$ (*c* 1.41, $CHCl_3$). MS (FAB) *m/z* 603 [M + H$^+$]. Anal. calcd for $C_{29}H_{38}N_2O_4S_3Si$: C, 57.77; H, 6.35; N, 4.65. Found: C, 57.88; H, 6.31; N, 4.48.

D-α-10a. Colourless oil. MS (FAB) *m/z* 603 [M + H$^+$]. Anal. calcd for $C_{29}H_{38}N_2O_4S_3Si$: C, 57.77; H, 6.35; N, 4.65. Found: C, 57.99; H, 6.24; N, 4.79.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-5-fluorouracil (L-β-9b) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-L-*erythro*-pentofuranosyl]-5-fluorouracil (L-α-9b). These compounds were obtained in 54 and 10% yield, respectively, starting from **8a** and 5-fluorouracil after silica gel column chromatography (CH_2Cl_2/MeOH, 94/6) followed by preparative TLC (CH_2Cl_2/MeOH, 92/8).

L-β-9b. White solid, mp 73–76 °C (dec). R_f 0.5. $[\alpha]_D^{20} = -33.6$ (*c* 0.69, $CHCl_3$/MeOH, 3/2). IR ($CHCl_3$): 3012, 2926, 1703, 1465, 1427 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.01 (d, *J* = 6.0 Hz, 1H), 7.67–7.62 (m, 4H), 7.47–7.33 (m, 6H), 6.42–6.35 (m, 1H), 4.47–4.45 (m, 1H), 4.06 (dd, *J* = 11.8, 1.6 Hz, 1H), 3.69 (dd, *J* = 11.7, 2.8 Hz, 1H), 3.08–2.90 (m, 1H), 2.25–2.20 (m, 1H), 2.08–2.01 (m + s, 1H + 9H), 1.08 (s, 9H). MS (FAB) *m/z* 621 [M + H$^+$]. Anal. calcd for $C_{29}H_{37}FN_2O_4S_3Si$: C, 56.10; H, 6.01; N, 4.51. Found: C, 56.27; H, 6.12; N, 4.69.

L-α-9b. Colourless oil. R_f 0.5. ^1H NMR ($CDCl_3$): δ 7.87 (d, *J* = 6.6 Hz, 1H), 7.67–7.62 (m, 4H), 7.47–7.33 (m, 6H), 6.42–6.35 (m, 1H), 4.47–4.45 (m, 1H), 3.92 (dd, *J* = 1.8, 11.1 Hz 1H), 3.65 (dd, *J* = 2.8, 11.5 Hz, 1H), 3.20–3.11 (m, 1H), 3.08–2.90 (m, 1H), 2.67–2.60 (m, 1H), 2.12 (s, 9H), 1.07 (s, 9H). MS (FAB) *m/z* 621

[M + H$^+$]. Anal. calcd for $C_{29}H_{37}FN_2O_4S_3Si$: C, 56.10; H, 6.01; N, 4.51. Found: C, 56.31; H, 6.13; N, 4.37.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-5-fluorouracil (D-β-10b) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-D-*erythro*-pentofuranosyl]-5-fluorouracil (D-α-10b). Prepared in 53 and 10% yield, respectively, as described for L-β-**9b** starting from **8b** and 5-fluorouracil.

D-β-10b. White solid, mp 75–77 °C (dec); $[\alpha]_D^{20} = +39.2$ (*c* 0.71, $CHCl_3$/MeOH, 3/2). MS (FAB) *m*/*z* 621 [M + H$^+$]. Anal. calcd for $C_{29}H_{37}FN_2O_4S_3Si$: C, 56.10; H, 6.01; N, 4.51. Found: C, 56.41; H, 6.13; N, 4.25.

D-α-10b. MS (FAB) *m*/*z* 621 [M + H$^+$]. Anal. calcd for $C_{29}H_{37}FN_2O_4S_3Si$: C, 56.10; H, 6.01; N, 4.51. Found: C, 55.94; H, 6.22; N, 4.39.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-thymine (L-β-9c) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-L-*erythro*-pentofuranosyl]-thymine (L-α-9c). Prepared in 55 and 14% yield, respectively, starting from **8a** and thymine after purification by silica gel chromatography ($CHCl_3$/MeOH, 99/1) followed by preparative TLC (five runs, $CHCl_3$/MeOH, 98/2).

L-β-9c. White solid, mp 56 °C (dec); R_f 0.3; $[\alpha]_D^{20} = -52.0$ (*c* 2.50, $CHCl_3$). IR ($CHCl_3$): 4214, 3379, 3009, 1689 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 8.55 (br s, 1H), 7.66–7.63 (m, 4H), 7.57 (s, 1H), 7.45–7.27 (m, 6H), 6.44 (dd, *J* = 6.5, 8.0 Hz, 1H), 4.47–4.46 (m, 1H), 4.09 (dd, *J* = 1.3, 11.6 Hz, 1H), 3.75 (dd, *J* = 2.2, 11.2 Hz 1H), 3.17–3.06 (m, 1H), 2.88 (ddd, *J* = 1.6, 5.9, 13.8 Hz, 1H), 2.12 (s, 9H), 2.05–1.93 (m, 1H), 1.59 (s, 3H), 1.09 (s, 9H). MS (FAB) *m*/*z* 617 [M + H$^+$]. Anal. calcd for $C_{30}H_{40}N_2O_4S_3Si$: C, 58.41; H, 6.54; N, 4.54. Found: C, 58.25; H, 6.69; N, 4.39.

L-α-9c. Colourless oil. R_f 0.4. ^{1}H NMR ($CDCl_3$): δ 9.05 (bs, 1H), 7.66–7.59 (m, 4H), 7.63 (s, 1H), 7.46–7.32 (m, 6H), 6.42–6.39 (m, 1H), 4.48–4.46 (m, 1H), 3.91 (dd, *J* = 10.9, 1.9 Hz, 1H), 3.67 (dd, *J* = 12.6, 2.2 Hz, 1H), 3.13–3.11 (m, 1H), 2.59–2.52 (m, 1H), 2.33–2.17 (m, 1H), 2.12 (s, 9H), 1.93 (s, 3H), 1.07 (s, 9H). MS (FAB) *m*/*z* 617 [M + H$^+$]. Anal. calcd for $C_{30}H_{40}N_2O_4S_3Si$: C, 58.41; H, 6.54; N, 4.54. Found: C, 58.64; H, 6.43; N, 4.73.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-thymine (D-β-10c) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-D-*erythro*-pentofuranosyl]-thymine (D-α-10c). Prepared in 60 and 14% yield, respectively, starting from **8b** and thymine as described for L-β-**9c**.

D-β-10c. $[\alpha]_D^{20} = +25.0$ (*c* 1.90, $CHCl_3$). MS (FAB) *m*/*z* 617 [M + H$^+$]. Anal. calcd for $C_{30}H_{40}N_2O_4S_3Si$: C, 58.41; H, 6.54; N, 4.54. Found: C, 58.25; H, 6.68; N, 4.72.

D-α-10c. MS (FAB) *m*/*z* 617 [M + H$^+$]. Anal. calcd for $C_{30}H_{40}N_2O_4S_3Si$: C, 58.41; H, 6.54; N, 4.54. Found: C, 58.09; H, 6.33; N, 4.75.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-cytosine (L-β-9d) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-L-*erythro*-pentofuranosyl]-cytosine (L-α-9d). Obtained by reacting **8a** with cytosine in 51 and 4.5% yield, respectively, after purification by silica gel chromatography ($CHCl_3$/MeOH, 9/1) followed by preparative TLC (six runs, $CHCl_3$/MeOH, 9/1).

L-β-9d. White solid, mp 96 °C (dec); R_f 0.5. $[\alpha]_D^{20} = -59.5$ (*c* 0.42, $CHCl_3$). IR ($CHCl_3$): 3419, 3022, 2399, 2346, 1643, 1478 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.97 (d, *J* = 7.2 Hz, 1H), 7.64–7.61 (m, 4H), 7.40–7.32 (m, 6H), 6.37–6.31 (m, 1H), 5.38 (d, *J* = 7.5 Hz, 1H), 4.45–4.44 (m, 1H), 4.06 (dd, *J* = 1.5, 11.6 Hz, 1H), 3.78 (dd, *J* = 2.8, 11.6, 1H), 3.05–2.89 (m, 2H), 2.25–1.73 (m + s, 1H + 1H), 1.06 (s, 9H). MS (FAB) *m*/*z* 602 [M + H$^+$]. Anal. calcd for $C_{29}H_{39}N_3O_3S_3Si$: C, 57.87; H, 6.53; N, 6.98. Found: C, 57.65; H, 6.70; N, 6.62.

L-α-9d. Colourless oil. R_f 0.4. ^{1}H NMR ($CDCl_3$): δ 7.76 (d, *J* = 6.9 Hz, 1H), 7.66–7.60 (m, 4H), 7.46–7.32 (m, 6H), 6.34–6.15 (m, 1H), 5.75 (d, *J* = 6.9 Hz, 1H), 4.48 (m, 1H), 3.89 (dd, *J* = 11.1, 2.5 Hz, 1H), 3.67 (dd, *J* = 10.9, 2.6 Hz, 1H), 3.05–2.92 (m, 2H), 2.09 (s, 10H), 1.04 (s, 9H). MS (FAB) *m*/*z* 602 [M + H$^+$]. Anal. calcd for $C_{29}H_{39}N_3O_3S_3Si$: C, 57.87; H, 6.53; N, 6.98. Found: C, 58.25; H, 6.43; N, 7.30.

1-[5′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-cytosine (D-β-10d) and 1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-α-D-*erythro*-pentofuranosyl]-cytosine (D-α-10d). Prepared in 57 and 5% yield, respectively, as described for L-β-**9d**.

D-β-10d. White solid, mp 94 °C (dec); $[\alpha]_D^{20} = +58.4$ (*c* 1.12, $CHCl_3$). MS (FAB) *m*/*z* 602 [M + H$^+$]. Anal. calcd for $C_{29}H_{39}N_3O_3S_3Si$: C, 57.87; H, 6.53; N, 6.98. Found: C, 58.15; H, 6.33; N, 7.22.

D-α-10d. Colourless oil. MS (FAB) *m*/*z* 602 [M + H$^+$]. Anal. calcd for $C_{29}H_{39}N_3O_3S_3Si$: C, 57.87; H, 6.53; N, 6.98. Found: C, 58.22; H, 6.32; N, 7.28.

General procedure for the synthesis of nucleosides L-β-3a–d and D-β-4a–d

A solution of **9** or **10** (0.12 mmol) in dry THF (1 mL) was treated with a 1 M solution of TBAF in THF (0.28 mmol) and the mixture was stirred at room temperature for 1 h. Evaporation of the solvent under reduced pressure gave the crude nucleoside.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-uracil (L-β-3a). This compound was obtained in 95% yield starting from L-β-**9a** as a white solid after purification by silica gel chromatography ($CHCl_3$/MeOH, 9/1): mp 212–214 °C; $[\alpha]_D^{20} = -19.1$ (*c* 0.31, $CHCl_3$/MeOH, 3/2). IR ($CHCl_3$): 3013, 2392, 1687, 1518, 1462 cm^{-1}. ^1H NMR ($CDCl_3$ + few drops of CD_3OD): δ 7.69 (d, *J* = 8.0 Hz, 1H), 6.25–6.15 (dd, *J* = 6.6, 13.3 Hz, 1H), 5.67 (d, *J* = 8.1, 1H), 4.45–4.43 (m, 1H), 4.01 (dd, *J* = 2.1, 11.3 Hz, 1H), 3.83 (dd, *J* = 2.3, 11.0 Hz, 1H), 3.09–2.99 (m, 1H), 2.95–2.78 (m, 1H), 2.75–2.53 (m, 1H), 2.19 (s, 9H). MS (FAB) *m*/*z* 365 [M + H$^+$]. Anal. calcd for $C_{13}H_{20}N_2O_4S_3$: C, 42.84; H, 5.53; N, 7.69. Found: C, 43.01; H, 5.62; N, 7.44.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-uracil (D-β-4a). Prepared in 97% yield as described for L-β-**3a**: mp 213–214 °C; $[\alpha]_D^{20} = +21.3$ (*c* 0.81, $CHCl_3$/MeOH, 3/2). MS (FAB) *m*/*z* 365 [M + H$^+$]. Anal. calcd for $C_{13}H_{20}N_2O_4S_3$: C, 42.84; H, 5.53; N, 7.69. Found: C, 43.12; H, 5.43; N, 7.41.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-5-fluorouracil (L-β-3b). Starting from L-β-**9b** this compound was obtained in 72% yield as a white solid after purification by silica gel chromatography (CH_2Cl_2/MeOH, 92/8): mp 155–158 °C; $[\alpha]_D^{20} = -39.5$ (*c* 0.38, $CHCl_3$). IR ($CHCl_3$): 3378, 3020, 2362, 1707, 1422 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.39 (br s, 1H), 8.02 (d, *J* = 6.4 Hz, 1H), 6.29–6.22 (m, 1H), 4.49–4.45 (m, 1H), 4.05 (dd, *J* = 1.8, 11.4 Hz, 1H), 3.86 (dd, *J* = 2.3, 11.3 Hz, 1H), 3.09–3.01 (m, 1H), 2.99–2.82 (m, 1H), 2.19–2.11 (m + s, 1H + 9H). MS (FAB) *m*/*z* 383 [M + H$^+$]. Anal. calcd for $C_{13}H_{19}FN_2O_4S_3$: C, 40.82; H, 5.01; N, 7.32. Found: C, 41.00; H, 5.12; N, 7.44.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-5-fluorouracil (D-β-4b). Prepared in 81% yield as described for L-β-**3b**: mp 181–183 °C; $[\alpha]_D^{20} = +38.2$ (*c* 0.32, $CHCl_3$). MS (FAB) *m*/*z* 383 [M + H$^+$]. Anal. calcd for $C_{13}H_{19}FN_2O_4S_3$: C, 40.82; H, 5.01; N, 7.32. Found: C, 40.62; H, 4.90; N, 7.19.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-thymine (L-β-3c). This compound was obtained starting from L-β-**9c** in 90% yield as a white solid after purification by silica gel chromatography (CH_2Cl_2/MeOH, 94/6): mp 187 °C (dec); $[\alpha]_D^{20} = -10.4$ (*c* 0.48, $CHCl_3$). IR ($CHCl_3$): 4214, 3459, 3300, 3022, 1676, 1464 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.93 (br s, 1H), 7.44–7.38 (m, 1H), 6.21–6.13 (m, 1H), 4.40–4.38 (m, 1H), 3.99 (dd, *J* = 2.5, 11.6 Hz, 1H), 3.77 (dd, *J* = 3.2, 11.9 Hz, 1H), 3.12–3.00 (m, 1H), 2.97–2.71 (m, 1H), 2.34–2.18 (m + s, 1H + 9H), 1.88 (s, 3H). MS (FAB) *m*/*z* 379 [M + H$^+$]. Anal. calcd for $C_{14}H_{22}N_2O_4S_3$: C, 44.42; H, 5.86; N, 7.40. Found: C, 44.64; H, 5.89; N, 7.37.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-thymine (D-β-4c). Prepared in 98% yield as described for L-β-**3c**: mp 180–182 °C; $[\alpha]_D^{20} = +6.0$ (*c* 0.38, $CHCl_3$). MS (FAB) *m*/*z* 379 [M + H$^+$]. Anal. calcd for $C_{14}H_{22}N_2O_4S_3$: C, 44.42; H, 5.86; N, 7.40. Found: C, 44.57; H, 5.76; N, 7.22.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-cytosine (L-β-3d). Prepared in 90% yield starting from L-β-**9d**. White solid after purification by silica gel chromatography ($CHCl_3$/MeOH, 9/1): mp 69 °C (dec); $[\alpha]_D^{20} = -25.0$ (*c* 0.60, $CHCl_3$). IR ($CHCl_3$): 4214, 3406, 3327, 3009, 1650 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.77 (d, *J* = 7.2 Hz, 1H), 6.95 (br s, 1H), 6.15 (t, *J* = 6.4 Hz, 1H), 5.82 (d, *J* = 7.2 Hz, 1H), 4.48–4.43 (m, 1H), 3.95 (dd, *J* = 2.1, 10.9 Hz, 1H), 3.74 (dd, *J* = 1.8, 11.1 Hz, 1H), 3.31–3.12 (m, 1H), 3.11–2.92 (m, 1H), 2.87–2.68 (m, 1H), 2.15 (s, 9H). MS (FAB) *m*/*z* 364 [M + H$^+$]. Anal. calcd for $C_{13}H_{21}N_3O_3S_3$: C, 42.95; H, 5.82; N, 11.56. Found: C, 42.74; H, 5.90; N, 11.39.

1-[2′,3′-Dideoxy-3′-*C*-tris(methylthio)methyl-β-D-*erythro*-pentofuranosyl]-cytosine(D-β-4d). Prepared in 97% yield as described for L-β-**3d**: mp 72 °C (dec); $[\alpha]_D^{20} = +27.5$ (*c* 0.69, $CHCl_3$). MS (FAB) *m*/*z* 364 [M + H$^+$]. Anal. calcd for $C_{13}H_{21}N_3O_3S_3$: C, 42.95; H, 5.82; N, 11.56. Found: C, 42.72; H, 5.98; N, 11.69

***N*-Benzoyl-1-[5′-*O*-*tert*-butyldiphenylsilyl-2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-cytosine (12).** To a solution of **9d** (201 mg, 0.33 mmol) in anydrous DMF (5 mL) 4-nitrophenyl benzoate (121 mg, 0.49 mmol) and HOBt (23 mg, 0.16 mmol) was added and the reaction was stirred overnight at room temperature. The reaction was quenched with aqueous Na_2CO_3 (3 mL) and the mixture was extracted with CH_2Cl_2 (3×3 mL). The combined organic layers were then washed with small amounts of water (5×2 mL), brine (6 mL) and then dried over anydrous Na_2SO_4. Filtration and evaporation gave 250 mg of the crude product which was purified by column chromatography ($CHCl_3$/MeOH, 97/3) to give **12** (170 mg, 73%) as a white solid: mp 180 °C (dec); $[\alpha]_D^{20} = -29.1$ (*c* 0.24, $CHCl_3$). IR ($CHCl_3$): 3025, 2402, 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.36 (d, *J* = 7.6 Hz, 1H), 7.89 (d, *J* = 7.9 Hz, 1H + 1H), 7.65–7.37 (m, 14H), 6.36 (t, *J* = 6.2 Hz, 1H), 4.58–4.52 (m, 1H), 4.11–4.06 (m, 1H), 3.80 (dd, *J* = 3.2, 11.6 Hz), 3.23–3.11 (m, 1H), 2.97–2.86 (m, 1H), 2.17–2.00 (s + m, 9H + 1H), 1.09 (s, 9H). MS (FAB) *m*/*z* 707 [M + H$^+$]. Anal. calcd for $C_{36}H_{43}N_3O_4S_3Si$: C, 61.24; H, 6.14; N, 5.95. Found: C, 60.90; H, 6.20; N, 5.68.

***N*-Benzoyl-1-[2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-cytosine (13).** To a solution of **12** (163 mg, 0.23 mmol) in anydrous THF (1 mL) a 1 M solution of TBAF in THF (570 μL, 0.57 mmol) was added and the reaction was stirred for 2 h. The solvent was evaporated to give 380 mg of an orange oil which was purified by column chromatography ($CHCl_3$/MeOH, 95/5) to give **13** (81 mg, 75%) as a white solid:

mp 88 °C (dec); $[\alpha]_D^{20} = +19.0$ (*c* 0.21, $CHCl_3$). IR ($CHCl_3$): 3025, 2403, 1696 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.43 (d, *J* = 7.5 Hz, 1H), 7.82 (d, *J* = 7.3 Hz, 1H + 1H), 7.57–7.38 (m, 4H), 6.21 (t, *J* = 6.0 Hz, 1H), 4.57–4.55 (m, 1H), 4.10–4.04 (m, 1H), 3.87 (dd, *J* = 3.0, 12.0 Hz, 1H), 3.08–2.94 (m, 2H), 2.33–2.07 (m + s, 1H + 9H). MS (FAB) *m/z* 468 $[M+H^+]$. Anal. calcd for $C_{20}H_{25}N_3O_4S_3$: C, 51.37; H, 5.39; N, 8.99. Found: C, 51.72; H, 5.03; N, 9.32.

N-Benzoyl-1-[2′,3′-dideoxy-3′-*C*-tris(methylthio)methyl-β-L-*erythro*-pentofuranosyl]-cytosine-5′-(2-cyanoethyl hexadecyl phosphate) (16). To a solution of **14** (55 mg, 0.13 mmol) in anhydrous THF (1 mL) a solution of **13** (63 mg, 0.13 mmol) in anhydrous THF (1 mL) and a 0.45 M solution of tetrazole in CH_3CN (290 μL, 0.13 mmol) were added. The reaction mixture was stirred overnight at room temperature and then 0.1 M solution of ethyl-(methyl)dioxirane in CH_2Cl_2 (1.3 mL, 0.13 mmol) was added. After 2 h the solvent was removed to give 184 mg of yellow oil which was purified by column chromatography ($CHCl_3$/MeOH, 7/1) to afford **16** (25 mg, 23%) as a colourless oil: $[\alpha]_D^{20} = -20.0$ (*c* 0.50, $CHCl_3$). IR ($CHCl_3$): 3024, 2399, 1709 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.22 (d, *J* = 7.4 Hz, 1H), 7.90 (d, *J* = 7.2 Hz, 1H + 1H), 7.59–7.45 (m, 4H), 6.26 (t, *J* = 5.4 Hz, 1H), 4.72–4.62 (m, 1H), 4.35–4.20 (m, 2H), 4.08–4.13 (m, 2H), 3.19–3.02 (m, 1H), 3.82–3.01 (m, 1H), 2.8–2.71 (m, 2H), 2.20 (s, 9H), 2.04–2.12 (m, 1H), 1.58–1.63 (m, 2H), 1.24–1.22 (m, 28H), 0.87–0.78 (m, 3H). MS (FAB) *m/z* 826 $[M+H^+]$. Anal. calcd for $C_{39}H_{61}N_4O_7PS_3$: C, 56.77; H, 7.45; N, 6.79. Found: C, 57.05; H, 7.12; N, 7.12.

Ammonium salt of 1-[2′,3′-dideoxy-3′-*C*-tris(methylthio)-methyl-β-L-*erythro*-pentofuranosyl]-cytosine-5′-(hexadecyl phosphate) (11). Treatment of **16** (20 mg, 0.024 mmol) with concentrated aqueous ammonia (2.5 mL) overnight and purification by preparative TLC ($CHCl_3$/MeOH/NH_4OH, 12/4/1) gave **11** (5 mg, 30%) as a colourless oil. ^1H NMR (DMSO): δ 7.79 (d, *J* = 7.2 Hz, 1H), 6.25–6.17 (m, 1H), 5.97 (d, *J* = 7.1 Hz, 1H), 4.51–4.43 (m, 1H), 3.96–3.68 (m, 2H + 2H), 2.92–2.72 (m, 1H + 1H), 2.38–2.24 (m, 1H), 2.16 (s, 9H), 1.46–1.23 (m, 28H), 0.93–0.87 (m, 3H). MS (ES) *m/z* 666 $[M^+]$. Anal. calcd for $C_{29}H_{53}N_3O_6PS_3$: C, 51.37; H, 5.39; N, 8.99. Found: C, 51.70; H, 5.58; N, 8.68.

Biological studies

Thymidine kinase assay. HSV-1 TK was purified and assayed as previously described:[22] briefly, enzyme was incubated at 37 °C for 30 min in a mixture (25 μL) containing 30 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] K^+, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol (DTT), and 0.8 μM [^{3}H-methyl]-dThd (2200 cpm/pmol). The reaction was terminated by spotting 20 μL of the incubation mixture on a 25-mm DEAE paper disk (DE-81 paper, Whatman). The disk was washed with an excess of 1 mM ammonium formate (pH 3.6) in order to remove unconverted nucleoside, then with ethanol. [^{3}H]-TMP was estimated by scintillation counting in 1 mL of Betamax™ (ICN-Biomedicals).

When nucleoside analogues were tested as possible substrates of HSV-1 TK, 100μM of each compound was incubated at 37 °C for 20 min in in a mixture (25 μL) containing 30 mM HEPES K^+, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol (DTT) and 10 μg of HSV-1 TK. Samples were then heated at 100 °C for 5 min and centrifuged 15 min at 10,000 rpm in an Eppendorf bench fuge. Supernatants were transfered in a new tube for subsequent HPLC analysis.

HPLC separation of nucleosides and nucleotides

The reversed-phase chromatography method employing the HPLC system (SHIMADZU) was used in order to separate nucleosides from nucleotides. A 4.6 mm×25 cm ALLTIMA C18-NUC 100A 5U (Alltech) column was used at room temperature in the following conditions: injection volume, 20 μL; detection, UV 260 nm; eluents of the linear gradient (40 min): buffer A (20 mM KH_2PO_4, pH 7.5), buffer B (20 mM KH_2PO_4, pH 5.2, 60% methanol). Flow rate: 0.5 mL/min.

Cytotoxitity assay

Cell lines. All cell lines were obtained from ATCC. The cells were cultured in RPMI 1640 supplemented with 5% FCS, 0.1 mM glutamine, 1% penicillin and streptomycin. Cells were grown in Nunc clone plastic bottles (TedNunc, Roskilde, Denmark) and split twice weekly at different cell densities according to standard procedures. 3T3 cells were grown as monolayer and were split by using trypsin. Perypheral blood mononuclear cells (MNC) were separated from heparinized whole blood obtained from healthy donor on a Ficoll-Hypaque gradient as previously described.[27] MNC thus obtained were washed twice with RPMI 1640 supplemented with 10% FCS, glutamine and antibiotics, suspended at 200,000 viable cells/mL in medium containing, as mitogen, 5 μg/mL PHA (Sigma) and used in toxicity tests.

Chemicals. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma. It was dissolved at a concentration of 5 mg/mL in sterile PBS at room temperature and the solution was further sterilized by filtration and stored at 4 °C in a dark bottle. SDS was obtained from Sigma and DMF was purchaised from Fluka. Lysis buffer was prepared as follows: 20% w/v of SDS was dissolved at 37 °C in a solution of 50% of each DMF and demineralized water; pH was adjusted to 4.7 by adding 2.5% of an 80% solution of acetic acid and 2.5% 1 N HCl.

Toxicity tests. Cell were plated at different concentrations on flat-botton 96-well microplates (0.1 mL/well). Lymphocytes were plated out at 20,000 cells/well; 3T3 cells (murine fibroblast line) were plated at 10,000 cells/well; NSO cells (plasmocytoma murine cell line) were plated out at 3000 cells/well; Daudi cells (human lymphoblastoid cell line) were plated at 3000 cells/well. 12 h after plating, different concentrations of each compound were added to each well. After 48 h, MTT assay was performed to analyze cytotoxicity of the different compounds. Some experiments were performed by using confluent cells:

compounds were added on 3T3 monolayer 3 days after plating. Tests were then run as described above. Any compound was tested in triplicate and any experiment has been performed at least twice. Calculated ED_{50} values for NSO and normal lymphocytes are reported in Table 1. SD never excedeed 5% of the calculated dose. Results obtained were similar for all used lines.

MTT/formazan extraction procedure. 20 μL of the 5 mg/mL stock solution of MTT were added to each well; after 2 h of incubation at 37 °C, 100 μL of the extraction buffer were added. After an overnight incubation at 37 °C, the optical densities at 570 nm were measured using a Titer-Tech 96-well multiscanner, employing the extraction buffer as the blank. S.D. never exceded 5% of the mean value. The results obtained have been used to calculate ED_{50} values.

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