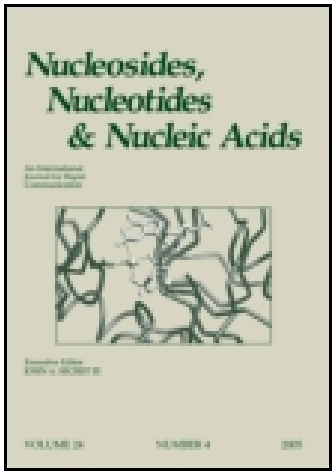

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Synthesis of Novel Acyclic Nucleoside Analogues with Anti-Retroviral Activity

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SYNTHESIS OF NOVEL ACYCLIC NUCLEOSIDE ANALOGUES WITH ANTI-RETROVIRAL ACTIVITY

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□ *A series of novel acyclic thymine nucleoside analogues were prepared by the Mitsunobu reaction from appropriately protected chiral triols. The enantiomeric triols were obtained from substituted γ-lactone acids, prepared by asymmetric oxidation of 3-substituted-1,2-cyclopentanediones. The cytotoxic activity of new analogues was evaluated on MCF-7 human breast cancer and HeLa cells, and antiviral activities on human immunodeficiency virus type 1 and hepatitis C virus models. The synthesized compounds revealed specific anti-retroviral activity and no cytotoxic side effects.*

Keywords Acyclic nucleosides; anti-retroviral activity; human immunodeficiency virus type 1; hepatitis C virus

1. INTRODUCTION

Acyclic nucleosides are a part of an important class of nucleosides that display remarkable biological activity. The discovery of acyclovir **1**[1] 20 years ago made this group of compounds especially attractive for researchers. This has led to the development of various new acyclic nucleoside structures (Figure 1; **2–4**) and methodologies for their synthesis.

Acyclic nucleoside analogues have shown activity against several viral infections, such as herpes simplex virus (HSV), human immunodeficiency virus (HIV), hepatitis B virus (HBV), and other infections.[2–5] Some acyclic nucleoside analogues have become popular drugs with wide clinical use.

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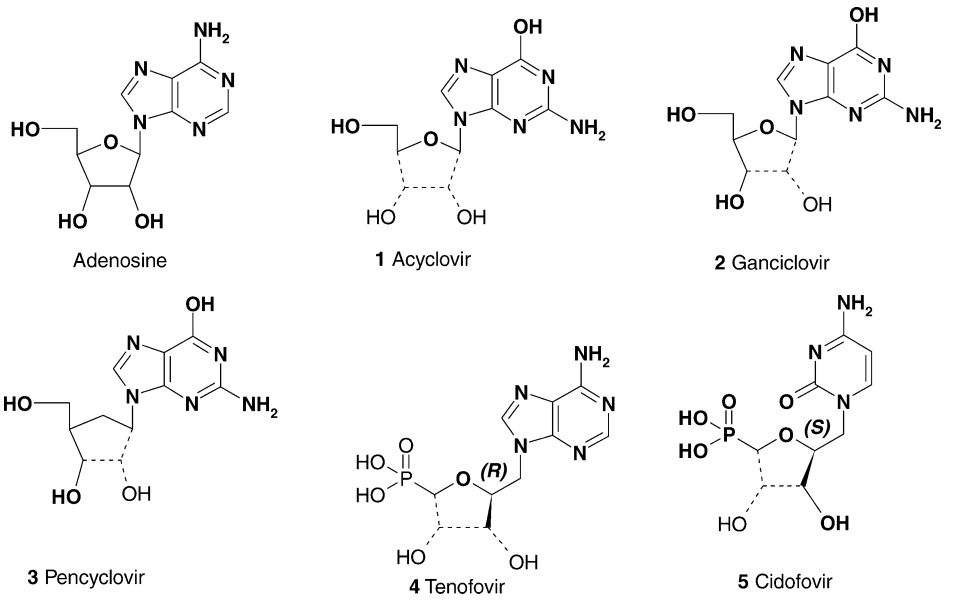


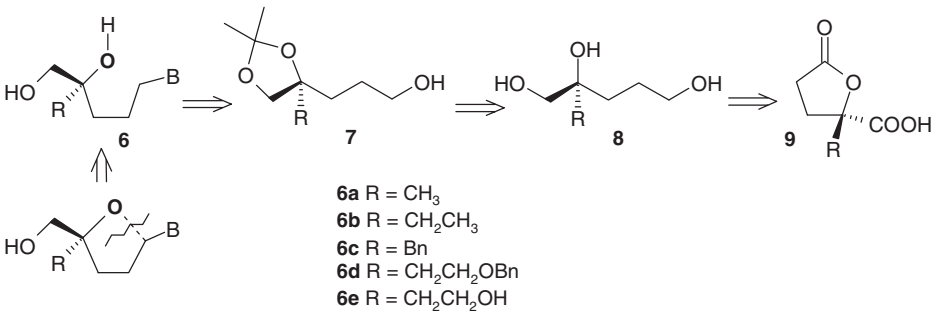
FIGURE 1 Some different structures of acyclic nucleosides. (Missing or changed adenosine fragment is designated with dotted line.)

Research is still intensively being undertaken to achieve new highly active compounds with low toxicity and minimal side effects.[6–10] The absolute configuration of the nucleoside analogues often play an important role in determining their biological activity and selectivity.[11–13] There are some enantiomeric acyclic nucleoside analogues that have already found clinical use: *(R)*-enantiomer of PMPA, tenofovir **4** is about 50 times more effective against HIV than its *(S)*-counterpart, whereas for the anti-human cytomegalovirus drug HPMPC, cidofovir **5**, the *(R)*-enantiomer is much less potent12[13] (Figure 1).

A number of stereoselective synthetic routes to acyclic nucleoside analogues in enantiomerically pure forms have been reported, including such starting from optically active natural compounds as carbohydrates,[14,15,] (+)-diethyl L-tartrate,[16] L-ascorbic and D-isoascorbic acids,[17] D-lactose,[18] or commercially available purine nucleosides.[19] Also, in some cases the methods of asymmetric synthesis have been applied: for example, Sharpless asymmetric dihydroxylation,[20,21] asymmetric Michael addition,[22] etc.

We have recently reported that optically pure 5-oxo-2-substituted tetrahydrofuran-2-carboxylic acids[23,24] are suitable precursors for the synthesis of 4′-alkyl– and 4′-aryl-2′,3′-dideoxynucleoside analogues.[25,26] The dideoxy analogues are known to possess antivirus activity[27–30] and, therefore, a further search for new compounds with improved selectivity and resistance profiles, as well as effectiveness against various drug-resistant reverse transcriptase variants, is the subject of extensive interest.

In this article, we describe a synthetic route to novel acyclic nucleosides, starting from enantiomeric 2-alkyl-substituted 2-hydroxyglutaric acid-γ-lactones[31] (Scheme 1).



SCHEME 1 Retrosynthetic route to acyclic 4′-alkyl-2′,3′-dideoxynucleoside analogues **6** from 2-alkyl-substituted 2-hydroxyglutaric acid-γ-lactones **9**.

These acyclic structures can be considered as 1′,4′-seco nucleoside analogues of the corresponding cyclic compounds. Indeed, the oxygen of the tertiary hydroxyl group of the structure **6** may act in a way similarly to the oxygen atom in a furanose ring. These compounds may benefit from the flexibility of the acyclic chain, which makes it possible to adopt the active site of the enzyme and, therefore, may have high potency against some drug-resistant viruses. It is reported that 4′-C-methyl and 4′-C-ethyl -2′-deoxy- nucleosides have revealed significant anti-HIV activity.[32,33] Also, the substituent R (substituted aryl- and hydroxyalkyl groups) in the compound may be used to tune properties of the compound, such as hydrophobicity, solubility, electronic properties, specific binding, etc.

2. RESULTS AND DISCUSSION

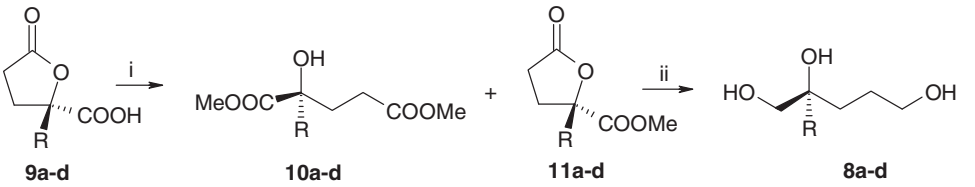
2.1. Synthesis of the Compounds

According to retrosynthetic analysis, the acyclic nucleoside analogues **6a–e** can be prepared directly by a coupling of enantiomeric acetals **7** with nucleobase B. Acetals **7** can be easily prepared from triols **8** via selective acetalization of the 1,2-diol moiety; triols **8** can be obtained from γ-lactone acids **9** (Scheme 1).

According to this general procedure, the starting compounds of the synthesis are γ-lactone acids **9a–d**. A general method for the synthesis of different enantio-enriched γ-lactone acids has been published by us previously.[31] The enantiomeric purity of crude compounds **9a** and **9c** was improved from 94–96% to $\geq$99%ee by recrystallization from an EtOAc-heptane mixture.

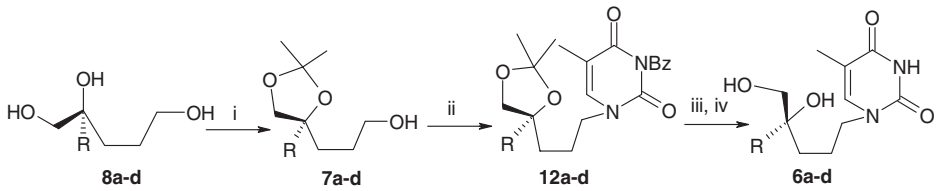
Compounds **9b** and **9d** were used directly as obtained from asymmetric oxidation reaction and chromatography on silica gel with 94% and 96%ee, correspondingly, without additional crystallization.

For the synthesis of triols **8,** lactone acids **9** were first converted to their methyl esters by refluxing in MeOH in the presence of a catalytic amount of concentrated HCl[34] to give a mixture of diesters **10** and lactone esters **11**. The structure of these esters, which were obtained in a ratio of 5:1, was confirmed by nuclear magnetic resonance (NMR) in the case of benzyl-substituted compounds **10c** and **11c** after chromatographical separation. In all other cases, the obtained crude mixture of esters **10** and **11** was reduced with $LiAlH_4$,[35] affording the corresponding diols **8a–d,** with 71–81% yield in two steps (Scheme 2).



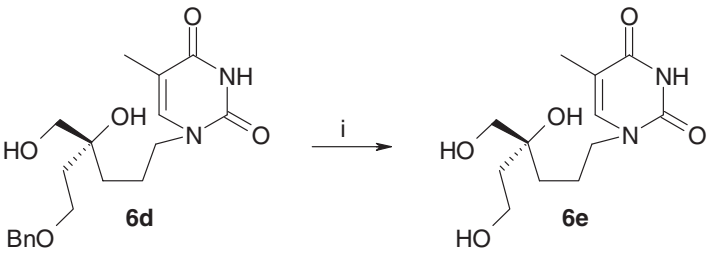
SCHEME 2 Synthesis of triols **8**. Reagents: (i) HCl/MeOH; (ii) $LiAlH_4$, THF.

Selective acetalization of 1,2-hydroxyl groups of triols **8** was carried out in acetone using the catalytic amount of *p*-TsOH, to afford acetals **7** in high yield (90–96%). For the direct coupling of acetals **7a–d** with N^3-benzoylthymine,[36] a Mitsunobu[37] reaction, which is widely applied for the synthesis of carbocyclic,[38–40] and also acyclic nucleosides,[41–43] was used. Thus, the coupling of acetals **7** with N^3-Bz-thymine, followed by the sequential treatment of compounds **12** with methanolic ammonia and hydrochloric acid, produced the target acyclic nucleoside analogues **6a-d,** with a 65–80% yield, in three steps (Scheme 3).



SCHEME 3 Synthesis of acyclic nucleoside analogues **6a–d**. Reagents: (i) Acetone, p-TsOH; (ii) N^3-Bz-thymine, PPh_3, DEAD THF; (iii) NH_3/MeOH, (iv) HCl/MeOH/H_2O.

In the case of compound **6d**, the benzyl protecting group was removed by reduction with H_2 on Pd-catalyst, affording the acyclic nucleoside analogue **6e,** in 89% yield (Scheme 4).



SCHEME 4 Synthesis of acyclic nucleoside analogue **6e**. Reagents: (i) H_2, 10%Pd/C, MeOH.

2.2. Biological Testing: Cytotoxicity of the Compounds

Two different assays were used to detect the cytotoxic activity of the synthesized nucleoside analogues. First, a cell proliferation assay using the MCF-7 cells was carried out. (The results are presented in Table 1). As is evident from the obtained data, this analysis failed to detect any cytotoxic or cytostatic effect by day 9, while cell viability in the presence of 2 μM Camptothecin or 1 μM Gemcitabine was estimated as 2% or 5%, respectively. Next, the cytotoxicity of the compounds was determined using HeLa cells and an MTT cell viability assay. The normalized results of this assay are shown in Table 1.

As is evident from this data, none of the compounds caused cell death or inhibited cell proliferation; the results of the MTT assay also confirmed that there was no reduction of the energy production (judged by the activity of mitochondrial dehydrogenases) in response to the treatment of the cells with novel nucleoside analogues. Thus, these compounds did not possess

TABLE 1 Results of the analysis of cytotoxic properties of compounds

		Cell viability	
No.	Compounds **6**	MCF-7*	HeLa**
1	**a**	119	97
2	**b**	119	104
3	**c**	129	110
4	**d**	102	108
5	**e**	117	103
6	**DMSO (control)**	100	100

*The number of viable cells in negative control samples (treated with DMSO alone) was taken as 100%. Cell viability is presented as a ratio (in percentage) of number of viable cells in experimental (treated with tested compound) to that in DMSO treated control cells.

**The $OD_{540\,nm}$ of DMSO-treated control cells was taken as 100%. Cell viability is presented as a ratio (in percentage) of the $OD_{540\,nm}$ of cells treated with the tested compounds to the $OD_{540\,nm}$ of DMSO-treated cells.

cytotoxic or cytostatic properties; if anything, the treatment of cells with these compounds slightly activated cell division and energy metabolism.

2.3. Biological Testing: Anti-Retroviral Activities of the Compounds

The anti-retroviral activity of synthesized nucleoside compounds was tested using human immunodeficiency virus type 1 (HIV-1)-based virus-like particles (VLPs), prepared by use of the Invitrogen ViraPower Lentiviral Expression System. These particles contained, along with HIV-1 reverse transcriptase, a recombinant RNA genome which, if reverse transcribed and integrated by viral enzymes, expresses resistance to the antibiotic blasticidin. Thus, the formation of colonies from VLP-infected cells in the presence of blasticidin depends on reverse transcription taking place in early stages of infection and can be used as a measure for the efficiency of reverse transcriptase inhibitors. Two well-characterized nucleoside inhibitors of HIV reverse transcriptase, Lam (Lamivudine, 2′,3′-dideoxy-3′-thiacytidine) and AZT (azidothymidine), were used as positive controls. The results of such analysis are shown in Table 2. These results are in agreement with known activities of these compounds. Importantly, all five tested acyclic compounds showed some inhibitory effect, although the effect was not as prominent as in the case of the positive controls (Table 2). Since the compounds lacked any cytotoxic side effects, the inhibition must have originated from the direct antiviral action of the compounds. The mechanisms of their action were not studied in detail. However, action as chain-terminators, typical for acyclic compounds, represents the most likely option.

No acyclic compound synthesized in this study bore an -OH group which could be recognized as a 2′OH group of the ribose ring by RNA dependent RNA polymerases of viruses with RNA genomes. However, it was recently demonstrated that compounds without a 2′OH group,[42] or even acyclic

TABLE 2 Inhibition of activity of HIV reverse transcription by acyclic nucleosides and control substances (colony formation assay)

		Anti-retroviral viral activity	
No.	Compounds **6**	Number of colonies	Efficiency of colony formation (%)
	DMSO (control)	75	100
1	**a**	45	60
2	**b**	36	48
3	**c**	39	52
4	**d**	34	45
5	**e**	47	63
6	**Lam**	7	9
7	**AZT**	2	3

nucleosides,[43] can act as inhibitors of hepatitis C virus (HCV) RNA polymerase. To find out whether this was also the case for compounds described in this study, the anti-HCV activity was tested using an Huh7-Luc-neo/ET cell line, which carries a stably replicating HCV RNA replicon with an inserted firefly luciferase gene as a reporter.[44] In most cases, the compounds did not cause any reduction of HCV replication; in contrast, a slight activation was observed for compounds **6b–e** (data not shown). Interestingly, all these compounds also showed positive effects on cellular activities (Table 1). Thus, it is likely that the observed increase of HCV replication was mediated by the activation of cellular processes. The compound **6a** did not stimulate HCV replication, but its inhibitory effect on the HCV replication was very mild (inhibitory concentration 50 >100 μM). Thus, none of the acyclic compounds described in this study acted as inhibitors of HCV replication.

3. EXPERIMENTAL

3.1. General

^{1}H and ^{13}C spectra were determined in deuterated solvents on a Bruker AMX-500 spectrometer. Deuterated solvent peaks were used as references. Two-dimensional FT methods were used for the full assignment of ^{1}H and ^{13}C chemical shifts. Mass spectra were measured on a Hitachi M80B spectrometer, using an EI (70 eV) or Shimadzu GCMS–QP 2010 spectrometer, with EI (70 eV). Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum BX FTIR spectrometer. Elemental analyses were performed on a Perkin-Elmer C,H,N,S-Analyzer 2400 ga. Optical rotations were obtained using a Krüss Optronic GmbH polarimeter P 3002 and Anton Paar GWB polarimeter MCP 500. Thin layer chromatography (TLC) was performed using Merck DC-Alufolien Kieselgel 60 F_{254} silica gel plates. For column chromatography, silica gel KSK 40–100 μm was used. All reactions sensitive to oxygen or moisture were conducted under an argon atmosphere in oven-dried glassware. Commercial reagents were generally used as received. Tetrahydrofuran (THF) was distilled from $LiAlH_4$ before use.

General procedure for synthesis of triols 8a–d. A solution of lactone acid **9a** (720 mg, 5 mmol) and 1 drop of concentrated HCl in dry MeOH (15 mL) was heated to reflux overnight. After cooling, EtOAc (150 mL) was added and the mixture was washed with 5% $NaHCO_3$ (50 mL), brine (50 mL), and dried ($MgSO_4$). The solvents were removed under reduced pressure to yield 0.848 g of a crude mixture of esters. These esters were dissolved in dry THF (12 mL) and added a drop at a time at 0°C to a suspension of $LiAlH_4$ (339 mg, 8.9 mmol) in THF (12 mL). The mixture was heated to reflux for 3 hours. After cooling to 0°C, water (0.34 mL) was carefully added. The mixture was stirred at room temperature for 20 minutes, and 10% aqueous

NaOH (0.34 mL) was added a drop at a time, and stirred for another 20 minutes. Then water (1.02 mL) was added and the mixture was stirred for 30 minutes. After filtration with EtOAc (6 × 10 mL), the combined filtrate was dried (Na_2SO_4) and concentrated. The residue was purified by flash chromatography (silica gel, CH_2Cl_2/MeOH 30:1 to 10:1).

(S)-2-methylpentane-1,2,5-triol 8a. Obtained as a colorless oil (543 mg, 81%); $[\alpha]_D^{23}$ −1.7 (c 5.95, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$ + ΔCD_3OD): δ 4.55 (bs, 1H, 5-OH), 4.41 (bs, 1H, 1-OH), 4.17 (s, 1H, 2-OH), 3.47–3.52 (m, 2H, H-5), 3.26–3.33 (m, 2H, H-1), 1.40–1.56 (m, 4H, H-3,4), 1.04 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$ + ΔCD_3OD): δ 72.37 (C-2), 69.23 (C-1), 62.389 (C-5), 34.55 (C-3), 26.32 (C-4), 22.68 (CH_3); IR (neat, cm^{-1}): 3351, 2946, 2875, 1454, 1416, 1378, 1057, 635; MS (m/z): 103, 101, 85, 75, 57, 55, 43 (base). Calculated for $C_6H_{14}O_3$: C, 53.71; H, 10.52. Found: C, 53.58; H, 10.62.

(S)-2-ethylpentane-1,2,5-triol 8b. Obtained from **9b** (730 mg, 4.62 mmol) as a colorless oil (485 mg, 71%); $[\alpha]_D^{25}$ − 0.3 (c 13.0, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$ + ΔCD_3OD): δ 4.62 (bs, 1H, 5-OH), 4.35 (bs, 1H, 1-OH), 3.95 (s, 1H, 2-OH), 3.42 (J = 5.4 Hz, 2H, H-5), 1.29–1.43 (m, 6H, H-3,4,6), 0.71 (t, J = 7.5 Hz, 3H, H-7); ^{13}C NMR (125 MHz, $CDCl_3$ + ΔCD_3OD): δ 74.11 (C-2), 66.60 (C-1), 62.24 (C-5), 31.23 (C-3), 27.85 (C-6), 25.81 (C-4), 7.19 (C-7); IR (neat, cm^{-1}): 3351, 2944, 2880, 1462, 1337, 1137, 1057, 633; MS (m/z): 117, 101, 99, 89, 83, 71, 57 (base), 43. Calculated for $C_7H_{16}O_3$: C, 56.73; H, 10.88. Found: C, 56.71; H, 10.94.

(R)-2-benzylpentane-1,2,5-triol 8c. Obtained from **9c** (450 mg, 2.05 mmol) as a colorless oil, which solidified on standing (304 mg, 71%); $[\alpha]_D^{25}$ + 0.4 (c 8.12, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 7.27 (m, 2H, m-Ph), 7.22 (m, 2H, o-Ph), 7.21 (m, 1H, p-Ph), 3.57 (t, J = 5.8 Hz, 2H, H-5), 3.41 (s, 2H, H-1), 2.82 and 2.76 (2d, J = 13.5 Hz, 2H, Bn-CH_2), 1.67 (m, 2H, H-4), 1.51 (m, 2H, H-3); ^{13}C NMR (125 MHz, CD_3OD): δ 136.94 (s), 130.40 (o), 128.16 (m), 126.41 (p), 74.57 (C-2), 66.73 (C-1), 62.72 (C-6), 42.42 (Bn), 32.47 (C-3), 26.23 (C-4); IR (neat, cm^{-1}): 3368, 3086, 3062, 3029, 2947, 1603, 1495, 1454, 1055, 735, 703; MS (m/z): 179, 161, 151, 133, 119, 101 (base), 92, 91, 83. Calculated for $C_{12}H_{18}O_3$: C, 68.55; H, 8.63. Found: C, 68.46; H, 8.67.

(R)-2-(2-benzyloxyethyl)-pentane-1,2,5-triol 8d. Obtained from **9d** (1.87 g, 7.08 mmol) as a colorless oil (1.32 g, 73%); $[\alpha]_D^{23}$ − 0.8 (c 43.1, MeOH/$CHCl_3$ 1:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.28–7.36 (m, 5H, Ph), 4.50 (s, 2H, CH_2Ph), 3.95 (bs, OH), 3.53–3.70 (m, 6H, 2xOH, H-1, CH_2OBn), 3.45 (t, J = 5.0 Hz, 2H, H-5), 1.77–1.87 (m, 2H, CH_2CH_2OBn), 1.55–1.62 (m, 4H, H-3,4); ^{13}C NMR (125 MHz, $CDCl_3$): δ 137.39 (s-Ph), 128.43 (m-Ph), 127.84 (p-Ph), 127.74 (o-Ph), 73.79 (C-2), 73.33 (CH_2Ph), 67.54 (C-1), 66.57 (CH_2OBn), 62.78 (C-5), 35.96 (CH_2CH_2OBn), 33.82 (C-3), 26.25 (C-4); IR (neat, cm^{-1}): 3371, 3089, 3031, 296, 2872, 1954, 1586, 1496, 1454, 1058, 747, 699, 608; MS (m/z): 205, 195, 169, 159, 143, 129, 115, 107, 101, 99, 92, 91

(base), 79. Calculated for $C_{14}H_{22}O_4$: C, 66.12; H, 8.72. Found: C, 65.92; H, 8.78.

2-Benzyl-2-hydroxy-pentanedioic acid dimethyl ester 10c. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.14–7.29 (m, 5H, Ph), 3.72 (s, 3H, 1-OCH_3), 3.67 (s, 3H, 5-OCH_3), 3.17 (d, J = 0.9 Hz, 1H, 2-OH), 3.05 and 2.94 (2d, J = 13.5 Hz, 2H, CH_2Ph), 2.50–2.57 (m, 1H, H-4), 2.20–2.28 (m, 2H, H3, H4), 2.09–2.15 (m, 1H, H-3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.41 (C-1), 173.48 (C-5), 135.40 (s-Ph), 129.91 (o-Ph), 128.15 (m-Ph), 126.95 (p-Ph), 77.40 (C-2), 52.56 (1-OCH_3), 51.59 (5-OCH_3), 45.61 (CH_2Ph), 62.78 (C-5), 33.72 (C-3), 28.68 (C-4).

2-Benzyl-5-oxo-tetrahydrofuran-2-carboxylic acid methyl ester 11c. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.24–7.33 (m, 5H, Ph), 3.77 (s, 3H, OCH_3), 3.37 and 3.14 (2d, J = 14.4 Hz, 2H, CH_2Ph), 2.43–2.51 (m, 2H, H3, H-4), 2.22–2.30 (m, 1H, H3), 2.10–2.19 (m, 1H, H-4); ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.48 (C-5), 171.74 (2-COO), 133.83 (s-Ph), 130.48 (o-Ph), 128.54 (m-Ph), 127.45 (p-Ph), 86.26 (C-2), 52.92 (OCH_3), 42.46 (CH_2Ph), 29.93 (C-3), 27.96 (C-4).

General procedure for synthesis of acyclic nucleoside analogues 6a–d. A solution of triol **8a** (264 mg, 1.97 mmol) in acetone (10 mL) p-TsOH (3.8 mg, 0.02 mmol) was added. After stirring at room temperature for 4 hours, Et_3N (20 μL) was added, and the mixture was stirred for 5 minutes and concentrated. The residue was purified by flash chromatography (silica gel, petroleum ether/acetone 10:2), producing acetal **7a** (307 mg, 90%). Acetals **7b** (335 mg, 94%), **7c** (264 mg, 96%) and **7d** (730 mg, 94%) were obtained from triols **8b**.(280 mg, 1.89 mmol), **8c** (230 mg, 1.095 mmol), and **8d** (673 mg, 2.65 mmol), respectively.

To a solution of acetal **7a** (161 mg, 0.925 mmol), N ^{3}-benzoylthymine (253 mg, 1.1 mmol) and Ph_3P (288 mg, 1.1 mmol) in THF (19 mL) DEAD (173 μL, 1.1 mmol) were added dropwise at a time at 0°C. The mixture was stirred at room temperature overnight, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (silica gel and petroleum ether/acetone 10:2) to give **12a** (469 mg) as a crude white solid. This solid was dissolved in MeOH (2.8 mL), and saturated methanolic ammonia solution (2.8 mL) was added at 0°C. The mixture was stirred at room temperature overnight and the solvents were evaporated. The residue was dissolved in MeOH (2.8 mL), and water (0.93 mL) and 6N HCl solution (1.85 mL) were added. The mixture was stirred at room temperature for 4 hours and the solvents were evaporated. Flash chromatography on silica gel (CH_2Cl_2/MeOH 15:1 for **6a-b** and 30:1 to 20:1 for **6c-d)** gave the target compounds.

1-(4,5-Dihydroxy-4-methylpentyl)-5-methyl-1H-pyrimidine-2,4-dione 6a. Obtained as a white solid (177 mg, 79%); m.p. 169–172°C; $[\alpha]_D^{22}$ −1.2 (c 16.5, DMSO); ^{1}H NMR (500 MHz, CD_3OD): δ 7.43 (q, J = 1.0 Hz, 1H, H-6), 3.72 (t, J = 7.2 Hz, 2H, NCH_2), 3.34 (s, 2H, >C < CH_2OH), 1.86 (d, J = 1.0

Hz, 3H, 5-CH_3), 1.71–1.77 (m, 2H, $CH_2CH_2CH_2$), 1.43–1.52 (m, 2H, CH_2 > C<), 1.12 (s, 3H, CH_3 > C<); ^{13}C NMR (125 MHz, CD_3OD): δ 166.91 (C-4), 152.99 (C-2), 143.22 (C-6), 111.09 (C-5), 73.28 (COH), 70.24 (CH_2OH), 49.80 (NCH_2), 35.84 ($NCH_2CH_2CH_2$), 24.35 ($CH_2CH_2CH_2$), 23.78 (CH_3 > C<), 12.17 (5-CH_3); IR (KBr, cm^{-1}): 3354, 3260, 2988, 1674, 1466, 1424, 1350, 1285, 1218, 1104, 1054, 927, 762, 707; MS (m/z): 243, 227, 211, 206, 167, 152, 127, 109, 85 (base), 75, 57, 43. Calculated for $C_{11}H_{18}O_4N_2$: C, 54.53; H, 7.49; N, 11.56. Found: C, 54.03; H, 7.51; N, 11.59.

1-[4-Hydroxy-4-(hydroxymethyl)hexyl]-5-methyl-1H-pyrimidine-2,4-dione 6b. Obtained from **7b** (169 mg, 0.9 mmol) as a white solid (150 mg, 65%); m.p. 41–44°C; $\alpha]_D^{22}$ − 2.2 (c 12.9, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 7.43 (q, J = 1.0 Hz, 1H, H-6), 3.71 (t, J = 7.1 Hz, 2H, NCH_2), 3.37 (s, 2H, > C < CH_2OH), 1.85 (d, J = 1.0 Hz, 3H, 5-CH_3), 1.67–1.73 (m, 2H, $CH_2CH_2CH_2$), 1.43–1.51 (m, 4H, CH_2 > C < CH_2), 0.85 (t, J = 7.4 Hz, 3H, CH_3CH_2); ^{13}C NMR (125 MHz, CD_3OD): δ 166. 87 (C-4), 152.95 (C-2), 143. 26 (C-6), 111.04 (C-5), 75.22 (COH), 67.36 (> C < CH_2OH), 49.81 (NCH_2), 33.14 ($NCH_2CH_2CH_2$), 29.52 (> C < CH_2CH_3), 24.00 ($CH_2CH_2CH_2$), 12.18 (5-CH_3), 7.86 (> C < CH_2CH_3); IR (KBr, cm^{-1}): 3419, 2942, 1680, 1472, 1358, 1218, 1050, 904, 783, 766; MS (m/z): 256, 238, 225, 197, 167, 152, 127, 112, 99 (base), 96, 83, 57, 41. Calculated for $C_{12}H_{20}O_4N_2$: C, 56.24; H, 7.87; N, 10.93. Found: C, 55.89; H, 7.88; N, 10.84.

1-(4-Benzyl-4,5-dihydroxypentyl)-5-methyl-1H-pyrimidine-2,4-dione 6c. Obtained from **7c** (225 mg, 0.9 mmol) as a white solid (230 mg, 80%); m.p. 94–96°C; $[\alpha]_D^{23}$ + 3.1 (c 23.5, MeOH/$CHCl_3$ 1:1); ^{1}H NMR (500 MHz, CD_3OD): δ 7.34 (q, J = 1.0 Hz, 1H, H-6), 7.19–7.23 (m, 4H, o-,m-Ph), 7.13–7.18 (m, 1H, p-Ph), 3.68 (t, J = 7.0 Hz, 2H, NCH_2), 3.36 ja 3.33 (2d, J = 11.0 Hz, 2H, CH_2OH), 2.78 and 2.74 (2d, J = 13.5 Hz, 2H, CH_2Ph), 1.83 (d, J = 1.0 Hz, 3H, 5-CH_3), 1.74–1.82 (m, 2H, $CH_2CH_2CH_2$), 1.40 (ddd, J = 5.7, 10.8 and 13.7 Hz, 1H, $NCH_2CH_2CH_2$), 1.35 (ddd, J = 6.2, 11.3 and 13.7 Hz, 1H, $NCH_2CH_2CH_2$); ^{13}C NMR (125 MHz, CD_3OD): δ 166.89 (C-4), 152.94 (C-2), 143.31 (C-6), 138.69 (s-Ph), 131.62 (o-Ph), 128.92 (m-Ph), 127.19 (p-Ph), 110.93 (C-5), 75.45 (COH), 67.28 (CH_2OH), 49.75 (NCH_2), 43.30 (CH_2Ph), 33.21 ($NCH_2CH_2CH_2$), 24.01 ($CH_2CH_2CH_2$), 12.22 (5-CH_3); IR (KBr, cm^{-1}): 3418, 3030, 2949, 1674, 1473, 1357, 1218, 1100, 1056, 704; MS (m/z): 300, 287, 272, 227 (base), 209, 195, 168, 161, 140, 127, 115, 101, 91, 83, 65, 55, 41. Calculated for $C_{17}H_{22}O_4N_2$: C, 64.13; H, 6.97; N, 8.80. Found: C, 63.92; H, 6.93; N, 8.76.

1-[6-Benzyloxy)-4-hydroxy-4-(hydroxymethyl)hexyl]-5-methyl-1H-pyrimidine-2,4-dione 6d. Obtained from **7d** (368 mg, 1.25 mmol) as a white solid (343 mg, 76%); m.p. 45–48°C; $[\alpha]_D$−2.2 (c 10.1, MeOH/$CHCl_3$ 1:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.87 (s, 1H, NH), 7.26–7.35 (m, 5H, Ph), 6.96 (q, J = 1.1 Hz, 1H, H-6), 4.49 (s, 2H, $BnCH_2$), 3.62–3.68 (m, 5H, NCH_2, $BnOCH_2$ and OH), 3.41–3.49 (m, 3H, > C < CH_2O and OH), 1.87 (d, J = 1.1 Hz, 3H, 5-CH_3), 1.77–1.85 (m, 2H, $BnOCH_2CH_2$), 1.68–1.79

(m, 2H, NCH_2CH_2), 1.47–1.54 (m, 2H, $NCH_2CH_2CH_2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 164.54 (C-4), 151.18 (C-2), 140.53 (C-6), 137.46 (s-Ph), 128.44 (m-Ph), 127.85 (p-Ph), 127.76 (o-Ph), 110.55 (5-CH_3), 73.80 (>C<), 73.36 ($BnCH_2$), 67.53 (> C < CH_2OH), 66.55 ($BnOCH_2$), 48.80 (NCH_2), 35.93 ($BnOCH_2CH_2$), 33.54 ($NCH_2CH_2CH_2$), 23.12 ($CH_2CH_2CH_2$), 12.15 (5-CH_3); IR (KBr, cm^{-1}): 3386, 3032, 2950, 1674, 1474, 1365, 1213, 1104, 1051, 741, 700; MS (m/z): 362, 331, 313, 271, 256, 238, 223, 207, 182, 167, 152, 127, 109, 91 (base), 79, 65, 45, 41. Calculated for $C_{19}H_{26}O_5N_2$: C, 62.97; H, 7.23; N, 7.73. Found: C, 62.74; H, 7.44; N, 7.68.

1-[4,6-dihydroxy-4-(hydroxymethyl)hexyl]-5-methyl-1H-pyrimidine-2,4-dione 6e. To a solution of nucleoside analogue **6d** (240 mg, 0.66 mmol) in MeOH (20 mL), 10% Pd/C (93 mg) was added. Through the reaction mixture, H_2 was bubbled at room temperature for an hour and a half. The catalyst was removed by filtration and the filtrate was evaporated. The residue was purified by flash chromatography (silica gel, CH_2Cl_2/MeOH 15:1 to 10:1) affording **3e** as a white solid (180 mg, 89%); m.p. 119–121°C; $[\alpha]_D^{23}$ − 1.9 (c 12.2, MeOH); ^1H NMR (500 MHz, CD_3OD): δ 7.43 (q, J = 1.0 Hz, 1H, H-6), 3.72 (t, J = 7.4 Hz, 2H, NCH_2), 3.70 (t, J = 6.8 Hz, 2H, CH_2CH_2OH), 3.40 and 3.39 (2d, J = 11.0 Hz, 2H, CH_2OH), 1.86 (d, J = 1.0 Hz, 3H, 5-CH_3), 1.71–1.77 (m, 4H, $CH_2CH_2CH_2$ and CH_2CH_2OH), 1.47–1.55 (m, 2H, $NCH_2CH_2CH_2$); ^{13}C NMR (125 MHz, CD_3OD): δ 166.90 (C-4), 152.98 (C-2), 143.23 (C-6), 111.08 (C-5), 74.81 (COH), 68.05 (CH_2OH), 58.90 (CH_2CH_2OH), 49.75 (NCH_2), 39.62 (CH_2CH_2OH), 34.43 ($NCH_2CH_2CH_2$), 24.03 ($CH_2CH_2CH_2$), 12.20 (5-CH_3); IR (KBr, cm^{-1}): 3414, 3168, 3047, 2921, 1690, 1474, 1355, 1218, 1047, 873, 757; MS (m/z): 272, 241, 236, 223, 206, 167, 152, 127, 115, 110, 97 (base), 83, 69, 55, 41. Calculated for $C_{12}H_{20}O_5N_2$: C, 52.93; H, 7.40; N, 10.29. Found: C, 52.81;, H, 7.37; N 10.22.

Procedure for testing cytotoxic activity. A cell proliferation assay was carried out, using the MCF-7 cells. Compounds were dissolved in DMSO and added to the cells at a final concentration of 100 μM (the concentration of DMSO was 0.1% in all cases). Cells were incubated in the presence of the compound and the number of living cells was counted after incubation for 3, 6, and 9 days. Camptothecin 2 μM and Gemcitabine 1 μM and 10 μM were used as a positive (cytotoxic) control and PBS + 0.1% DMSO as a negative control. The analysis was carried out at AS InBio (Tallinn, Estonia).

The cytotoxicity of the compounds was determined using HeLa cells and an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay. Cells were seeded on 96-well plates and incubated with 50 μM concentrations of the tested substances for 9 days. Cells treated with 0.5% of DMSO (the same amount of DMSO was used in experimental samples) were used as controls. Every 3 days, the media were replaced with fresh ones containing the same concentration of the compounds. After 9 days, an MTT reagent was added to the media at 10% concentration, cells were incubated

at 37°C for 2 hours, lysed in DMSO, and the optical density at 540 nm was measured.

Procedure for testing anti-retroviral activity. The anti-retroviral activity of synthesized nucleoside compounds was tested using HIV-1-based virus-like particles (VLPs) (ViraPower Lentiviral Expression System, Invitrogen). Nucleoside inhibitors of HIV reverse transcriptase, lamivudine, 2′,3′-dideoxy-3′-thiacytidine (Lam) and azidothymidine (AZT) were used as positive controls. Briefly, U2OS cells seeded on 60 mm plates at ~95% of confluency were infected with 75 cfu (colony forming units) of HIV-1-based VLPs, in the presence of 50 μM of tested/control substances and 6 μg/μL polybrene. Cells used as negative control were treated with DMSO (the same amount as was used in experimental cells). Twenty-four hour postinfection blasticidin selection (5 μg/mL) was applied. Ten days after infection, the cells were stained with crystal violet and the antibiotic resistant colonies were counted. Experiment was repeated three times with similar results. The number of colonies on the plates treated with DMSO alone (negative control) was taken as 100%. In the case of the positive controls AZT and Lam, the number of the formed colonies was reduced 35 and 10 times, respectively.

Procedure for testing anti-HCV-activity. The Huh-luc/neo-ET cells (derived from a human hepatocarcinoma cell line) seeded on 60 mm diameter dishes were incubated with 100 μM concentrations of the substances for 56 hours (control cells were incubated with the appropriate amount of DMSO), then lysed, and firefly luciferase activity in the lysates was measured according to the protocol of the Promega Luciferase Assay Systems. The total protein concentration in the lysates was determined using a Bio-rad Bradford assay and luminescence was normalized to the total protein concentration. This analysis was carried out at Baltic Technology Development (Tallinn, Estonia).

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