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Original article

Synthesis and molecular modelling of unsaturated exomethylene pyranonucleoside analogues with antitumor and antiviral activities

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Abstract

This report describes the total and facile synthesis of the unsaturated keto and exomethylene pyranonucleoside analogues, 1-(2,3,4-trideoxy-4-methylene-6-*O*-trityl-α-D-*glycero*-hex-2-enopyranosyl)uracil (**10**), 1-(2,3-dideoxy-α-D-*glycero*-hex-2-enopyranosyl-4-ulose)uracil (**17**) and 1-(2,3,4-trideoxy-4-methylene-α-D-*glycero*-hex-2-enopyranosyl)uracil (**18**). Commercially available 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**1**) was condensed with silylated uracil, deacetylated and acetalated to afford 1-(2,3-*O*-isopropylidene-α-D-mannopyranosyl)uracil (**4**). Two different synthetic routes were investigated for the conversion of **4** into the olefinic derivative 1-(2,3,4-trideoxy-4-methylene-6-*O*-trityl-α-D-*glycero*-hex-2-enopyranosyl)uracil (**10**). Although the two procedures are quite similar with respect to yields and final products, the second also leads to the keto-2′,3′-unsaturated analogue (**17**). The new analogues were evaluated for their anticancer and antiviral activities using several tumor cell lines and gastrointestinal rotavirus. All of the compounds showed direct antiviral effect against rotavirus infectivity in Caco-2 cell line. Moreover, 1-(2,3,4-trideoxy-4-methylene-6-*O*-trityl-α-D-*glycero*-hex-2-enopyranosyl)uracil (**10**) was found to be potent in MCF-7 breast carcinoma cell line.

Keywords: Unsaturated nucleosides; Exomethylene nucleosides; Ketonucleosides; Antiviral; Cytotoxicity; MCF-7 cell line

1. Introduction

Nucleoside analogues display a wide range of biological activities, such as antitumor, antiviral and chemotherapeutic [1–4]. Over the past two decades, nucleoside chemistry has evolved to facilitate efficient routes to effective agents for the treatment of AIDS [5], herpes [6] and viral hepatitis [7].

Lately, nucleosides bearing a six-membered carbohydrate moiety have been evaluated for their potential antiviral [8–11] and antibiotic [12] properties and as building blocks in nucleic acid synthesis [13,14]. In particular, six-membered carbocyclic nucleosides show resistance to hydrolysis, since glycosidic bond cleavage is a frequently encountered degradative pathway of nucleoside antivirals, especially for the 2′,3′-dideoxy-nucleosides [15]. On the other hand, the insertion of unsaturation in the 2′,3′-position of the glycone moiety to provide 2′,3′-didehydro–2′,3′-dideoxy analogues distinguishes this ring system from common six-membered rings and allows to categorize the cyclohexene ring as a bioisoster of a saturated furanose ring [16]. Among them, the ketonucleosides proved to be key intermediates in synthetic and biosynthetic processes and a number of them exhibit interesting antitumor and antiviral properties [17–21]. It appears that these nucleosides not only exhibit growth inhibitory activity against a variety of tumor cells [22,23] *in vitro* and L1210 leukemia [24,25] *in vivo*, but they also may constitute important synthetic intermediates in nucleoside chemistry due to their chemical reactivity in various media [26,27]. Furthermore, the presence of an α,β-unsaturated double

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bond enhances biological activity [19,24,28], and these unsaturated ketonucleosides are well established for their antineoplastic activity and immunosuppressive properties [18,29,30].

Furthermore, modified nucleosides bearing an exocyclic methylene or fluoromethylene group in positions 2′, 3′ or 4′ exhibited noteworthy antitumor and antiviral activities [31–40]. Their antiviral and anticancer activities were attributed, at least in part, to the ability of these nucleosides to irreversibly inactivate ribonucleotide reductase after phosphorylation *via* specific nucleoside kinases.

Recently, we reported [41] on a new class of unusual nucleosides, both 2′-exomethylene and 3′,4′-unsaturated-2′-exomethylene pyranonucleosides, with satisfactory rotavirus and antitumor growth inhibition.

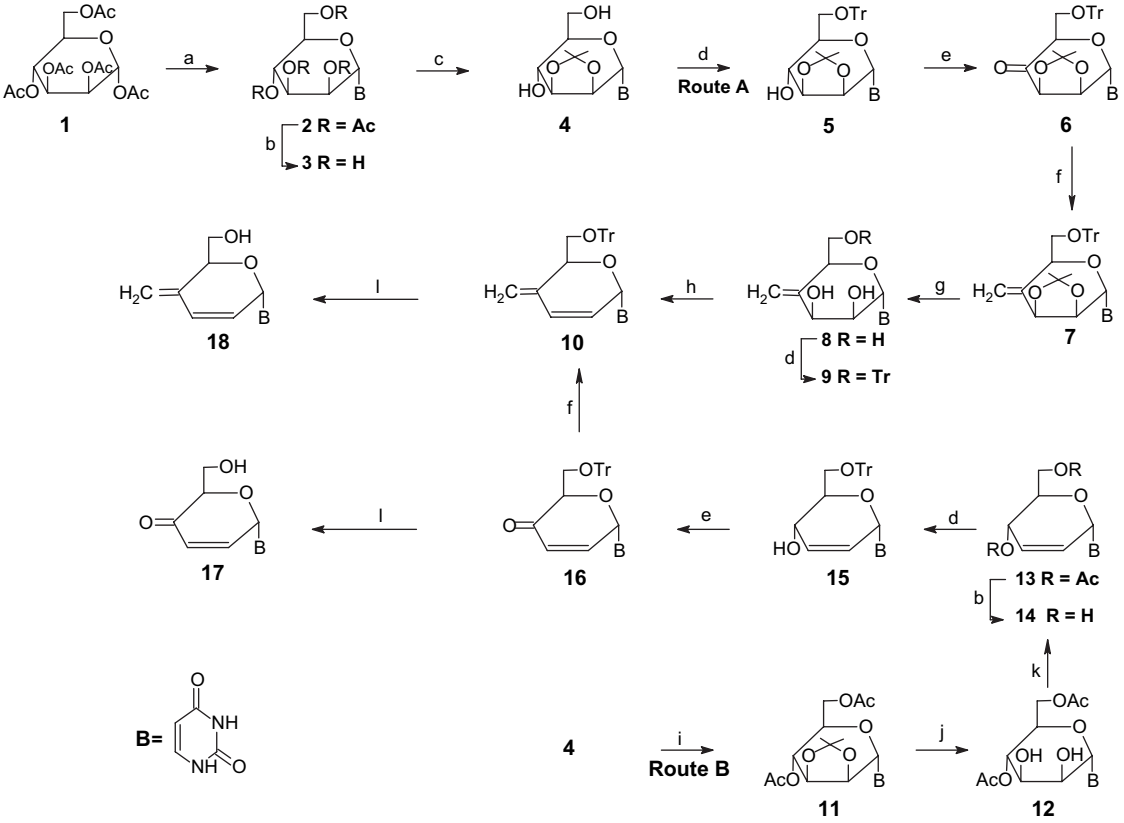
On the basis of these findings, we report herein the synthesis and pharmacology of a novel series of modified nucleosides, *i.e.* the 4′-exomethylene and 2′,3′-unsaturated-4′-exomethylene pyranonucleosides shown in Scheme 1.

2. Results and discussion

2.1. Synthesis

Condensation of commercially available 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**1**) with silylated uracil in the presence of trimethylsilyl trifluoromethane-sulfonate [42] ($Me_3SiOSO_2CF_3$) afforded 1-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)uracil (**2**). Removal of all *O*-acetyl protecting groups of **2**, with saturated methanolic ammonia [43], gave **3**, which was selectively acetylated, using 2,2-dimethoxypropane [43] in acetone, to give 2′,3′-*O*-isopropylidene **4**.

Two routes were investigated for the conversion of 1-(2,3-*O*-isopropylidene-α-D-mannopyranosyl)uracil (**4**) into the olefinic derivative **10**. In the first approach, the primary hydroxyl group of **4** was selectively tritylated to give **5**. Oxidation of the free hydroxyl group with the pyridinium dichromate (PDC)/acetic anhydride (Ac_2O) [44] led to the formation of the desired 4′-ketonucleoside **6**, which was isolated as a white solid. The ^{1}H NMR spectrum of **6** showed a doublet at 4.65 ppm ($J_{2',3'} = 7.4$ Hz), which is assigned to H-3′ confirming the absence of proton at C-4′. Wittig olefination of the keto intermediate **6** with sodium hydride and methyl triphenylphosphonium bromide (Ph_3PCH_3Br) at 0 °C, in the presence of *t*-amyl alcohol [38] in tetrahydrofuran (THF), led to **7**, which was obtained as a white solid in satisfactory yield (73%). The ^{1}H NMR spectrum of **7** showed two vinylic protons at 5.40 and 5.26 ppm, each as a broad singlet, which correspond to the 4′-exomethylene group. Compound **7** was converted to the fully unprotected derivative **8** by treatment with trifluoroacetic acid (TFA) 90% in methanol (MeOH), for 10 min. The primary hydroxyl group of **8** was selectively tritylated to afford derivative **9** in 78% yield. The olefination of the 2,3-vicinal diol



Scheme 1. (a) Uracil, $Me_3SiOSO_2CF_3$, HMDS, saccharine, dry CH_3CN, 120–80 °C; (b) ammonia/MeOH; (c) acetone, 2,2-dimethoxypropane, *p*-toluenesulfonic acid, water; (d) triphenylmethyl chloride, pyridine; (e) PDC, Ac_2O, dry CH_2Cl_2; (f) Ph_3PCH_3Br, sodium hydride, *t*-amyl alcohol in THF, 0–25 °C; (g) TFA 90% in MeOH; (h) dry Tol/DMF (4:1), iodine–imidazole–Ph_3P, 80 °C; (i) pyridine, Ac_2O; (j) formic acid/CH_2Cl_2 (1:1); (k) dry Tol/DMF (4:1), iodoform–imidazole–Ph_3P, 120 °C; and (l) formic acid/diethyl ether, 1:1.

proved to be a crucial step in our synthetic methodology and several possible synthetic approaches to the olefinic nucleoside **10** were investigated. Thus, we employed the Corey–Winter [45,46] on diol **9** by reacting it with thiocarbonyldiimidazole followed by reductive olefination with trimethylphosphite, the Barton [47] deoxygenation *via* the corresponding xanthate ester (treatment with tributyltin hydride), and the Hanessian et al. [48] method, which involved treatment with *N*,*N*-dimethylformamide (DMF) and dimethylacetal/methyl iodide. None of these methods were successful, as only intractable were isolated. In contrast, the Garegg–Samuelsson method (iodine–triphenylphosphine (Ph_3P)–imidazole [49–52]) led to the direct conversion of diol **9** to the desired olefinic compound 10 in 54% yield. The ^{1}H NMR spectrum of **10** showed, besides the two exomethylene protons at 5.15 and 5.04 ppm, the presence of two vinylic protons at 5.69 and 6.62 ppm (doublet, $J_{2',3'} = 9.1$ Hz) ascribed to H-2′ and H-3′, respectively.

In the second approach, acetylation of **4** using acetic anhydride in pyridine followed by deisopropylidenation of the resulted derivative **11** in dichloromethane/formic acid, 1:1, led to the vicinal diol derivative **12** in high yield (88%). Olefination of **12** with iodoform–Ph_3P–imidazole [53–55] afforded 1-(4,6-di-*O*-acetyl-2,3-dideoxy-α-D-*erythro*-hex-2-enopyranosyl)uracil (**13**) in 76% yield. The ^{1}H NMR spectrum of **13** showed the presence of two vinylic protons, H-2′ appearing as a doublet of doublets at 5.91 ppm ($J_{1',2'} = 1.5$ Hz, $J_{2',3'} = 10.1$ Hz) and a doublet at 6.34 ppm, which is assigned to H-3′. Deacetylation of **13** with saturated methanolic ammonia gave **14**, which after selective tritylation of the primary hydroxyl group afforded **15**. Oxidation of the free hydroxyl with PDC/Ac_2O and Wittig condensation of 4′-ketonucleoside **16** led to the 1-(2,3,4-trideoxy-4-methylene-6-*O*-trityl-α-D-*glycero*-hex-2-enopyranosyl)uracil (**10**). Finally, detritylation of **16** and **10** with formic acid/diethyl ether [56], 1:1, for 7 min, afforded the 4′-keto-2′,3′-unsaturated derivative **17** as well as the title nucleoside 1-(2,3,4-trideoxy-4-methylene-α-D-*glycero*-hex-2-enopyranosyl)uracil (**18**), respectively (Scheme 1).

In conclusion, the yields and the experimental processes quoted above are quite similar, although the second approach is more attractive because it leads not only the olefinic 4′-exomethylene derivative **10** but also the 4′-keto-2′,3′-unsaturated nucleoside **17.**

2.2. Conformational analysis of synthesized exomethylene pyranonucleoside analogues using computational methods

In an effort to decipher the preferred low energy conformations of the new keto and exomethylene pyranonucleoside analogues and also correlate their 3D-structures with the ^{1}H NMR spectrum, we studied the conformational preferences of **8**, **10**, **17** and **18**. The study was mainly focused on the formation of uracil nucleobase in correlation with the sugar moiety using energy minimization methods and systematic Grid Scan search.

Fig. 1 shows the lowest and the highest energy conformers of **8**, **10**, **17** and **18** after Grid Scan. In analogue **8** and in agreement with the ^{1}H NMR data (Table 1), the base moiety occupies the equatorial position (energetically favoured). The $H_{1'}$ and $H_{2'}$ protons of **8** have an axial–axial direction with a dihedral angle of $H_{2'}$–$C_{2'}$–$C_{1'}$–$H_{1'}$: 178.1° and $J_{1',2'} = 9.5$ Hz (Table 1) adopting thus a ''chair'' conformation. Moreover, in all analogues the low energy difference revealed that after rotation around $C_{1'}$–N_1 and $C_{5'}$–$C_{6'}$ bonds (Grid Scan simulation) the positions of the base and hydroxy methyl groups are possibly interconverted at room temperature (Fig. 1). Finally, computational analysis and ^{1}H NMR data of analogues **10** (dihedral angle $H_{2'}$–$C_{2'}$–$C_{1'}$–$H_{1'}$: 102.0°, $J_{1',2'} \sim 0$), **17** (dihedral angle $H_{2'}$–$C_{2'}$–$C_{1'}$–$H_{1'}$: 86.7°, $J_{1',2'} \sim 0$) and **18** (dihedral angle $H_{2'}$–$C_{2'}$–$C_{1'}$–$H_{1'}$: 103.1°, $J_{1',2'} \sim 0$) suggest that these compounds adopt the ''sofa'' conformation with H-1′ proton lying perpendicularly to the ring (Fig. 1). Such a conformation, compared to the ''chair'' conformation of analogue **8**, should be favoured because of the insertion of a double bond in the 2′,3′-position of the sugar moiety.

2.3. Biological activity

2.3.1. Antiviral activity

The antiviral properties of the nucleoside analogues were examined using a colon adenocarcinoma Caco-2 cell line infected with gastrointestinal rotavirus as a model virus and AZT drug as positive control (Table 2). Although the tested compounds did not show any antiviral activity in neutralization assay, they inhibited rotavirus infectivity following virus attachment. In comparison to AZT, higher concentrations were needed in order to obtain the same effect against virus and their CC_{50}/IC_{50} values were smaller than that found for AZT.

2.3.2. Cytotoxic and growth inhibition activity

The cytotoxicity (CC_{50}) of the new compounds was measured on normal human intestinal cell line (H4) and on a series of human tumor cells, such as human colonic adenocarcinoma cell line (Caco-2), skin melanoma cell line, and epithelial breast cancer cell line (MCF-7). The growth inhibition of Caco-2 cells induced by the new compounds was measured by determining the minimal inhibitory concentration, IC_{50}. The results are summarized in Table 3 and compared with the values obtained with 5-fluorouracil (5FU).

All of the tested compounds exhibit higher cytotoxicity in tumor cells than in the normal H4 cell line. Compound **10** showed to be 3-fold more cytotoxic to skin melanoma and MCF-7 cell line than to Caco-2 cells. Furthermore, compared to 5FU compound **10** was found to be 16-fold more selective toward MCF-7 cells, 2.5-fold more selective in Caco-2 cells and as selective as 5FU in skin melanoma cells. Compounds **8**, **17** and **18** in comparison to 5FU exhibit higher selectivity in all tested tumorgenic cell lines and higher cytotoxicity toward Caco-2 and MCF-7 cell lines. This selective activity of the tested compounds is of great importance in their potential use as antitumor drugs, as nucleoside analogues are molecules that require specialized nucleoside transporter proteins to

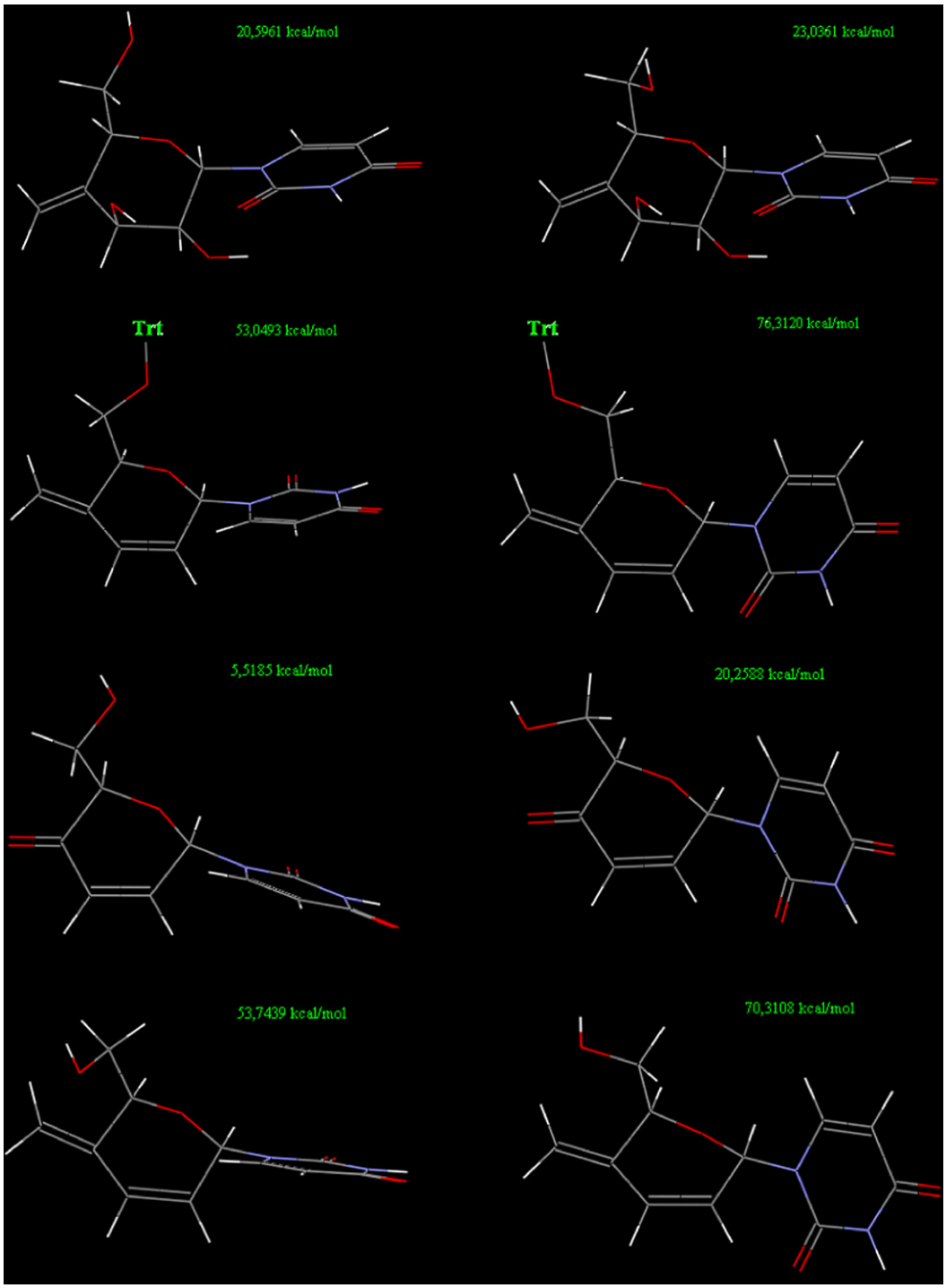


Fig. 1. The lowest (left side) and the highest energy (right side) conformations of **8**, **10**, **17** and **18** analogues obtained after energy minimization and Grid Scan simulation.

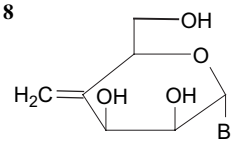
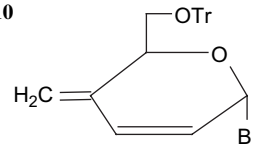
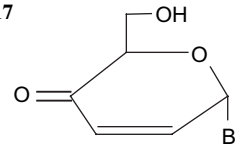
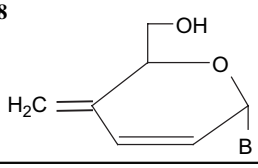
enter the cell and there is emerging evidence that the abundance and tissue distribution of these proteins contributes to cellular specificity and sensitivity to nucleoside analogues [57].

Furthermore, the pronounced inhibitory activity of the new compounds and especially of analogue **10** on cell growth in Caco-2 cells (2.5-fold higher TSI values than 5FU) identifies them as potential lead candidates for *in vivo* experiments.

3. Conclusion

In conclusion, biological assays were performed on the new compounds using a variety of tumor cell lines. 1-(2,3,4-Trideoxy-4-methylene-6-*O*-trityl-α-D-*glycero*-hex-2-enopyranosyl)uracil (**10**) being sufficiently cytotoxic on carcinoma cells and also highly selective was identified as a lead compound for further studies. All new molecules inhibited the growth of

Table 1
Torsion angles and *J* coupling of analogues **8**, **10**, **17** and **18** obtained from the 3D-structures and ^{1}H NMR, respectively

Analogue	Torsion angles	*J* Coupling (Hz)
8 	$C_{4'}-C_{5'}-C_{6'}-O_{6'}$: 177.7°	–
	$H_{3'}-C_{3'}-C_{2'}-H_{2'}$: 52.0°	–
	$H_{2'}-C_{2'}-C_{1'}-H_{1'}$: 178.1°	9.5
	$C_{2'}-C_{1'}-N_1-C_6$: −112.9°	–
10 	$C_{4'}-C_{5'}-C_{6'}-O_{6'}$: 163.7°	–
	$H_{3'}-C_{3'}-C_{2'}-H_{2'}$: −6.3°	9.1
	$H_{2'}-C_{2'}-C_{1'}-H_{1'}$: 102.0°	0
	$C_{2'}-C_{1'}-N_1-C_6$: 120.0°	–
17 	$C_{4'}-C_{5'}-C_{6'}-O_{6'}$: −179.5°	–
	$H_{3'}-C_{3'}-C_{2'}-H_{2'}$: −20.0°	8.9
	$H_{2'}-C_{2'}-C_{1'}-H_{1'}$: 86.7°	0
	$C_{2'}-C_{1'}-N_1-C_6$: 90.0°	–
18 	$C_{4'}-C_{5'}-C_{6'}-O_{6'}$: 44.9°	–
	$H_{3'}-C_{3'}-C_{2'}-H_{2'}$: −7.6°	10.1
	$H_{2'}-C_{2'}-C_{1'}-H_{1'}$: 103.1°	0
	$C_{2'}-C_{1'}-N_1-C_6$: 100.0°	–

Caco-2 cells and showed direct antiviral effect as they were able to inhibit rotavirus action.

4. Experimental

4.1. General procedure

Thin-layer chromatography (TLC) was performed on Merck precoated $60F_{254}$ plates. Reactions were monitored by TLC on silica gel, with detection by UV light (254 nm) or by charring with sulphuric acid. Flash chromatography was performed using silica gel (240–400 mesh, Merck).

^{1}H NMR spectra were recorded at room temperature with a Bruker 400 MHz spectrometer using chloroform-*d* ($CDCl_3$) and methanol-d_4 (CD_3OD). Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane (TMS) as internal standard. Mass spectra were obtained with a Micromass Platform LC (ESI-MS). Optical rotations were measured using a Schmidt and Haensch polarimeter. All reactions were carried out in dry solvents. CH_2Cl_2 was distilled from phosphorous pentoxide and stored over 4E molecular sieves. Acetonitrile, toluene (Tol) and DMF were distilled from calcium hydride and stored over 3E molecular sieves. THF was freshly distilled under nitrogen from sodium/benzophenone before use and pyridine stored over pellets of potassium hydroxide.

Table 2
Antiviral activity of nucleosides **8**, **10**, **17**, **18** and **AZT** against rotavirus RF strain on Caco-2 cells (IC_{50})

Compound	Treatment A[a]			Treatment B[a]		
	IC_{50}		CC_{50}/IC_{50}[b]	IC_{50}		CC_{50}/IC_{50}
	mg/mL	μM		mg/mL	μM	
8	n.e.	n.e.	–	0.2	740.00	0.1
10	n.e.[c]	n.e.	–	0.5	1044.8	0.04
17	n.e.	n.e.	–	0.2	839.60	0.1
18	n.e.	n.e.	–	0.2	846.60	0.1
AZT	0.02	74.84	0.75[d]	0.006	22.45	2.5[d]

[a] Treatment A: neutralization of the virus in the solution before its attachment. Treatment B: inhibition of infectivity following virus attachment.
[b] CC_{50}/IC_{50} values were calculated using CC_{50} values in Table 3.
[c] n.e. = No effect.
[d] CC_{50} for AZT on CaCo-2 cells = 56.1 μM.

Table 3
Cytotoxic effect (CC_{50}, μM) of compounds **8**, **10**, **17**, **18** and **5FU** on Caco-2, H4, MCF-7, and skin melanoma cells, and growth inhibition (IC_{50}, μM) on Caco-2 cells

Compound	Cytotoxic effect (CC_{50}, μM)				TSI[a]			Growth inhibition (IC_{50}, μM)
	H4	Caco-2	Melanoma	MCF-7	Caco-2	Melanoma	MCF-7	Caco-2
8	1850.2	74.0	74.0	74.0	25.0	25.0	25.0	7.4
10	1044.8	41.7	12.5	12.5	25.0	83.6	83.6	4.1
17	2099.0	83.9	83.9	83.9	25.0	25.0	25.0	8.3
18	2116.6	84.6	84.6	84.6	25.0	25.0	25.0	8.4
5FU	3843.8	384.4	46.1	768.8	10	83.4	5.0	1.5

[a] TSI: tumor selectivity index (CC_{50} on H4 cells/CC_{50} on the specified host cells).

4.1.1. 1-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl)uracil (2)

A mixture of uracil (4.96 g, 44.25 mmol), hexamethyldisilazane (HMDS) (11 mL, 52.75 mmol), and saccharin (0.37 g, 2.03 mmol) in dry CH_3CN (152 mL) was refluxed for 20 min at 120 °C under nitrogen. To this were added 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**1**) (12.32 g, 31.56 mmol) and $Me_3SiOSO_2CF_3$ (8 mL, 44.2 mmol). The reaction mixture was refluxed at 80 °C for 2 h. The mixture was neutralized with saturated sodium bicarbonate, then diluted with water, and extracted with ethyl acetate (EtOAc). The extract was dried with sodium sulfate, filtered and evaporated to a syrup, which was purified by flash chromatography (EtOAc/hexane, 7:3) to give **2** (12.56 g, 90%, $R_f = 0.44$ in EtOAc/hexane, 8:2) as a clear viscous oil. $[\alpha]_D^{22}$ 45.5 (*c* 0.230, $CHCl_3$); UV ($CHCl_3$): λ_{max} 261 nm (ε 7874); ^{1}H NMR ($CDCl_3$): δ 8.42 (br s, 1H, NH), 7.36 (d, 1H, $J_{5,6} = 8.2$ Hz, H-6), 6.14 (d, 1H, $J_{1',2'} = 9.6$ Hz, H-1′), 5.78 (d, 1H, H-5), 5.48 (t, 1H, $J_{2',3'} = J_{3',4'} = 3.2$ Hz, H-3′), 5.35 (dd, 1H, H-2′), 4.97 (d, 1H, H-4′), 4.63 (dd, 1H, $J_{5',6b} = 9.8$, $J_{6b',6a'} = 13.5$ Hz, H-6b′), 4.36–4.31 (m, 2H, $J_{5',6a'} = 5.3$ Hz, H-5′, H-6a′), 2.21, 2.18, 2.10, 1.99 (4s, 12H, 4OAc); ESI-MS (*m*/*z*) 443.45 [M + H]. Anal. Calcd. for $C_{18}H_{22}N_2O_{11}$: C, 48.87; H, 5.01; N, 6.33. Found: C, 48.95; H, 5.32; N, 6.21.

4.1.2. 1-(α-D-Mannopyranosyl)uracil (3)

Compound **2** (12.56 g, 28.42 mmol) was treated with ammonia/MeOH (saturated at 0 °C, 1 L). The solution was stirred

overnight at room temperature and then was concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc/MeOH, 8:2) to give **3** (6.80 g, 88%, $R_f = 0.24$) as a white foam. $[\alpha]_D^{22}$ 34.7 (*c* 0.150, MeOH); UV (MeOH): λ_{max} 260 nm (ε 6984); ESI-MS (*m/z*) 275.38 [M + H]. Anal. Calcd. for $C_{10}H_{14}N_2O_7$: C, 43.80; H, 5.15; N, 10.22. Found: C, 43.96; H, 5.32; N, 10.01.

4.1.3. 1-(2,3-O-Isopropylidene-α-D-mannopyranosyl)uracil (4)

To a stirred suspension of **3** (6.80 g, 25 mmol) in anhydrous acetone (50 mL) and 2,2-dimethoxypropane (50 mL) was added *p*-toluenesulfonic acid monohydrate (0.98 g, 5.15 mmol). After 30 min, water (100 mL) was added and the reaction mixture was stirred for 16 h. The resulting solution was neutralized with saturated sodium bicarbonate so that pH did not exceed 7, concentrated and the residue was purified by flash chromatography (EtOAc) to give **4** (6.30 g, 81%, $R_f = 0.14$) as a white foam. $[\alpha]_D^{22}$ 25.8 (*c* 0.280, MeOH); UV (MeOH): λ_{max} 260 nm (ε 6755); ^{1}H NMR ($CDCl_3$): δ 8.40 (br s, 1H, NH), 7.35 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 5.79 (d, 1H, H-5), 5.69 (d, 1H, $J_{1',2'} = 6.8$ Hz, H-1′), 4.55–4.47 (m, 2H, H-2′, H-3′), 4.22 (t, 1H, $J_{3',4'} = J_{4',5'} = 7.1$ Hz, H-4′), 3.87–3.76 (m, 3H, H-5′, H-6a′, H-6b′), 1.52, 1.39 (2s, 6H, $2CH_3$); ESI-MS (*m/z*) 315.34 [M + H]. Anal. Calcd. for $C_{13}H_{18}N_2O_7$: C, 49.68; H, 5.77; N, 8.91. Found: C, 49.45; H, 5.34; N, 8.63.

4.1.4. 1-(2,3-O-Isopropylidene-6-O-trityl-α-D-mannopyranosyl)uracil (5)

To a solution of **4** (1 g, 3.18 mmol) in pyridine (16 mL) were added triphenylmethyl chloride (1.15 g, 4.13 mmol) and a catalytic amount of 4-dimethylaminopyridine. The reaction mixture was stirred overnight at room temperature. After being quenched with MeOH and concentrated, the residue was purified by flash chromatography (EtOAc/hexane, 7:3) to give **5** (1.44 g, 82%, $R_f = 0.6$ in EtOAc) as a white solid, m.p. 143–146 °C. $[\alpha]_D^{22}$ 18.2 (*c* 0.100, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 8432); ^{1}H NMR ($CDCl_3$): δ 8.71 (br s, 1H, NH), 7.40 (d, 1H, $J_{5,6} = 8.2$ Hz, H-6), 7.32–7.21 (m, 15H, $3C_6H_5$), 5.75 (d, 1H, H-5), 5.70 (d, 1H, $J_{1',2'} = 6.5$ Hz, H-1′), 4.46–4.34 (m, 2H, H-2′, H-3′), 4.09 (m, 1H, H-4′), 3.84–3.77 (m, 1H, H-5′), 3.46–3.32 (m, 2H, H-6a′, H-6b′), 1.44, 1.31 (2s, 6H, $2CH_3$); ESI-MS (*m/z*) 557.81 [M + H]. Anal. Calcd. for $C_{32}H_{32}N_2O_7$: C, 69.05; H, 5.79; N, 5.03. Found: C, 69.21; H, 5.52; N, 5.21.

4.1.5. 1-(2,3-O-Isopropylidene-6-O-trityl-α-D-lyxo-hexopyranosyl-4-ulose) uracil (6)

A mixture of **5** (1.44 g, 2.61 mmol; dried by co-evaporation with Tol), PDC (1.17 g, 3.10 mmol) and Ac_2O (0.73 mL, 7.74 mmol) was stirred in dry CH_2Cl_2 (26 mL) for 4 h, under nitrogen at room temperature. Purification by flash chromatography (EtOAc/hexane, 8:2) yielded pure **6** (0.99 g, 70%, $R_f = 0.65$) as white solid, m.p. 115–118 °C. $[\alpha]_D^{22}$ 4.5 (*c* 0.150, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 7858); ^{1}H NMR ($CDCl_3$): δ 8.70 (br s, 1H, NH), 7.36 (d, 1H, $J_{5,6} = 8.2$ Hz, H-6), 7.24–7.15 (m, 15H, $3C_6H_5$), 5.72 (d, 1H, H-5), 5.56 (d, 1H, $J_{1',2'} = 3.6$ Hz, H-1′), 4.88 (dd, 1H, $J_{2',3'} = 7.4$ Hz, H-2′), 4.65 (d, 1H, H-3′), 4.09 (dd, 1H, $J_{5',6a'} = 2.6$, $J_{5',6b'} = 5.6$ Hz, H-5′), 3.64 (dd, 1H, $J_{6a',6b'} = 10.5$ Hz, H-6a′), 3.38 (dd, 1H, H-6b′), 1.48, 1.33 (2s, 6H, $2CH_3$); ESI-MS (*m/z*) 555.43 [M + H]. Anal. Calcd. for $C_{32}H_{30}N_2O_7$: C, 69.30; H, 5.45; N, 5.05. Found: C, 69.17; H, 5.21; N, 5.28.

4.1.6. 1-(4-Deoxy-2,3-O-isopropylidene-4-methylene-6-O-trityl-α-D-lyxo-hexopyranosyl)uracil (7)

To a stirred suspension of Ph_3PCH_3Br (2.11 g, 5.89 mmol) and *t*-amyl alcohol (0.71 mL, 6.44 mmol) in dry THF (17 mL) was added sodium hydride (0.15 g, 60% in oil, 6.44 mmol) at 0 °C under nitrogen and the reaction mixture was stirred for 2 h at ambient temperature under nitrogen. To this yellow phosphorous ylide was added a solution of **6** (0.99 g, 1.79 mmol) in dry THF (2.80 mL) dropwise, at 0 °C under nitrogen. The mixture was stirred for 30 min at ambient temperature under nitrogen, then the reaction mixture was quenched with saturated sodium bicarbonate and extracted with EtOAc. The organic layer was washed with water, dried with sodium sulfate and evaporated. The residue was purified by flash chromatography (EtOAc/hexane, 5:5) to give **7** (0.72 g, 73%, $R_f = 0.34$) as a white solid, m.p. 107–112 °C. $[\alpha]_D^{22}$ 29.8 (*c* 0.150, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 12,469); ^{1}H NMR ($CDCl_3$): δ 8.74 (br s, 1H, NH), 7.40 (d, 1H, $J_{6,5} = 8.1$ Hz, H-6), 7.31–7.19 (m, 15H, $3C_6H_5$), 5.80 (d, 1H, $J_{1',2'} = 6.7$ Hz, H-1′), 5.73 (d, 1H, H-5), 5.40, 5.26 (br s, 2H, methylene), 4.72 (d, 1H, $J_{2',3'} = 5.8$ Hz, H-3′), 4.56 (dd, 1H, H-2′), 4.21 (dd, 1H, $J_{5',6a'} = 5.5$, $J_{5',6b'} = 8.1$ Hz, H-5′), 3.62 (dd, 1H, $J_{6a',6b'} = 10.2$ Hz, H-6a′), 3.32 (dd, 1H, H-6b′), 1.36, 1.33 (2s, 6H, $2CH_3$); ESI-MS (*m/z*) 553.72 [M + H]. Anal. Calcd. for $C_{33}H_{32}N_2O_6$: C, 71.72; H, 5.84; N, 5.07. Found: C, 71.38; H, 5.65; N, 5.30.

4.1.7. 1-(4-Deoxy-4-methylene-α-D-lyxo-hexopyranosyl)uracil (8)

Compound **7** (0.72 g, 1.30 mmol) was dissolved in 5.85 mL of 90% TFA in MeOH. The solution was stirred for 10 min at room temperature and then concentrated under reduced pressure, in order to remove traces of TFA. The resulting residue was crystallized from diethyl ether to give pure **8** (0.30 g, 85%, $R_f = 0.4$ in EtOAc/MeOH, 9:1) as a white solid, m.p. 178–180 °C. $[\alpha]_D^{22}$ −72.7 (*c* 0.100, MeOH); UV (MeOH): λ_{max} 261 nm (ε 5418); ^{1}H NMR (CD_3OD): δ 7.67 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 6.15 (d, 1H, $J_{1',2'} = 9.5$ Hz, H-1′), 5.72 (d, 1H, H-5), 5.23, 5.15 (br s, 2H, methylene), 4.47–4.43 (m, 2H, H-2′, H-3′), 4.25 (dd, 1H, $J_{5',6'b} = 8.9$, $J_{6a',6b'} = 12.2$ Hz, H-6b′), 3.73 (dd, 1H, $J_{5',6a'} = 3.6$ Hz, H-5′), 3.53 (dd, 1H, H-6a′); ESI-MS (*m/z*) 271.32 [M + H]. Anal. Calcd. for $C_{11}H_{14}N_2O_6$: C, 48.89; H, 5.22; N, 10.37. Found: C, 48.57; H, 5.43; N, 10.21.

4.1.8. 1-(4-Deoxy-4-methylene-6-O-trityl-α-D-lyxo-hexopyranosyl)uracil (9)

To a stirred solution of **8** (0.30 g, 1.10 mmol) in pyridine (5 mL) were added successively triphenylmethyl chloride

(0.40 g, 1.43 mmol) and a catalytic amount of 4-dimethylaminopyridine. The reaction mixture was stirred overnight at room temperature, then was quenched with MeOH and evaporated under reduced pressure. The residue was purified by flash chromatography (EtOAc/hexane, 8:2) to give **9** (0.43 g, 78%, $R_f = 0.5$ in EtOAc) as a white foam. $[\alpha]_D^{22}$ 13.4 (*c* 0.200, $CHCl_3$); UV ($CHCl_3$): λ_{max} 261 nm (ε 8658); ^{1}H NMR ($CDCl_3$): δ 8.75 (br s, 1H, NH), 7.44 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 7.31–7.21 (m, 15H, $3C_6H_5$), 6.21 (d, 1H, $J_{1',2'} = 9.1$ Hz, H-1′), 5.75 (d, 1H, H-5), 5.22, 5.04 (br s, 2H, methylene), 4.54 (d, 1H, H-2′), 4.38 (br s, 1H, H-3′), 3.59–3.51 (m, 3H, H-5′, H-6a′, H-6b′); ESI-MS (*m/z*) 513.68 [M + H]. Anal. Calcd. for $C_{30}H_{28}N_2O_6$: C, 70.30; H, 5.51; N, 5.47. Found: C, 70.12; H, 5.70; N, 5.63.

*4.1.9. 1-(2,3,4-Trideoxy-4-methylene-6-O-trityl-α-D-glycero-hex-2-enopyranosyl)uracil (**10**)*

Imidazole (0.88 g, 1.29 mmol), Ph_3P (0.68 g, 2.58 mmol) and iodine (0.33 g, 1.29 mmol) were added to the suspension of **9** (0.44 g, 0.86 mmol) in 20 mL of dry Tol/DMF (4:1). The reaction mixture was heated (80 °C, oil bath) under nitrogen for 10 min. The residue was purified by flash chromatography (EtOAc/hexane, 5:5) to give **10** (0.22 g, 54%, $R_f = 0.6$ in EtOAc/hexane, 7:3) as a yellowish syrup. $[\alpha]_D^{22}$ 10.3 (*c* 0.125, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 6030); ^{1}H NMR ($CDCl_3$): δ 8.20 (br s, 1H, NH), 7.44 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 7.32–7.21 (m, 15H, $3C_6H_5$), 6.62 (d, 1H, $J_{2',3'} = 9.1$ Hz, H-3′), 6.58 (s, 1H, H-1′), 5.69 (m, 2H, H-2′, H-5), 5.15, 5.04 (br s, 2H, methylene), 4.47 (t, 1H, $J_{5',6a'} = J_{5',6b'} = 5.1$ Hz, H-5′), 3.49 (dd, 1H, $J_{6a',6b'} = 10.2$ Hz, H-6a′), 3.35 (dd, 1H, H-6b′); ESI-MS (*m/z*) 479.31 [M + H]. Anal. Calcd. for $C_{30}H_{26}N_2O_4$: C, 75.30; H, 5.48; N, 5.85. Found: C, 75.58; H, 5.63; N, 5.58.

*4.1.10. 1-(4,6-Di-O-acetyl-2,3-O-isopropylidene-α-D-manno-pyranosyl)uracil (**11**)*

Compound **4** (1.20 g, 3.82 mmol) was dissolved in a mixture of pyridine (14 mL) and Ac_2O (2 mL, 21.20 mmol). The reaction was carried out at room temperature for 2 h, then was quenched with MeOH at 0 °C and was concentrated. The residue was purified by flash chromatography (EtOAc/hexane, 7:3) to give **11** (1.26 g, 83%, $R_f = 0.6$ in EtOAc) as a viscous oil. $[\alpha]_D^{22}$ 14.7 (*c* 0.140, $CHCl_3$); UV ($CHCl_3$): λ_{max} 261 nm (ε 4326); ^{1}H NMR ($CDCl_3$): δ 9.05 (br s, 1H, NH), 7.30 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 5.78 (d, 1H, H-5), 5.72 (d, 1H, $J_{1',2'} = 5.5$ Hz, H-1′), 5.23 (dd, 1H, $J_{2',3'} = 6.5$ Hz, H-2′), 4.55–4.49 (m, 2H, H-3′, H-4′), 4.46–4.41 (m, 1H, H-5′), 4.18–4.10 (m, 2H, H-6a′, H-6b′), 2.12, 2.08 (2s, 6H, 2OAc), 1.58, 1.36 (2s, 6H, $2CH_3$); ESI-MS (*m/z*) 399.51 [M + H]. Anal. Calcd. for $C_{17}H_{22}N_2O_9$: C, 51.26; H, 5.57; N, 7.03. Found: C, 51.39; H, 5.38; N, 7.22.

*4.1.11. 1-(4,6-Di-O-acetyl-α-D-mannopyranosyl)uracil (**12**)*

Compound **11** (1.26 g, 3.18 mmol) was dissolved in a mixture of CH_2Cl_2 (11 mL) and formic acid (11 mL, 90%). The solution was stirred overnight at room temperature and then was concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc) to give **12** (1.0 g, 88%, $R_f = 0.25$) as a white solid, m.p. 118–121 °C; $[\alpha]_D^{22}$ 24.2 (*c* 0.250, $CHCl_3$); UV ($CHCl_3$): λ_{max} 261 nm (ε 4884); ^{1}H NMR ($CDCl_3$): δ 10.60 (br s, 1H, NH), 7.46 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 6.73 (d, 1H, H-5), 6.15 (d, 1H, $J_{1',2'} = 9.5$ Hz, H-1′), 5.04 (d, 1H, $J_{3',4'} = 3.4$ Hz, H-4′), 4.98 (t, 1H, $J_{2',3'} = 9.5$ Hz, H-2′), 4.28–4.14 (m, 3H, H-5′, H-6a′, H-6b′), 3.91 (dd, 1H, H-3′), 2.14, 2.05 (2s, 6H, 2OAc); ESI-MS (*m/z*) 359.42 [M + H]. Anal. Calcd. for $C_{14}H_{18}N_2O_9$: C, 46.93; H, 5.06; N, 7.82. Found: C, 46.72; H, 5.28; N, 7.69.

*4.1.12. 1-(4,6-Di-O-acetyl-2,3-dideoxy-α-D-erythro-hex-2-enopyranosyl)uracil (**13**)*

Imidazole (0.40 g, 5.88 mmol), Ph_3P (3.08 g, 11.75 mmol) and iodoform (2.31 g, 5.88 mmol) were added to the suspension of **12** (1 g, 2.80 mmol) in 40 mL of dry Tol/DMF (4:1). The reaction mixture was heated (120 °C, oil bath) under nitrogen for 2.5 h, was concentrated in vacuum and the residue diluted with EtOAc, washed with saturated sodium bicarbonate, sodium thiosulfate and water. The organic phase was dried with sodium sulfate, the solvent was removed in vacuum, and purification by flash chromatography (EtOAc /hexane, 6:4) yielded **13** (0.69 g, 76%, $R_f = 0.5$ in EtOAc), as a colorless oil. $[\alpha]_D^{22}$ 23.46 (*c* 0.115, $CHCl_3$); UV ($CHCl_3$): λ_{max} 261 nm (ε 5754); ^{1}H NMR ($CDCl_3$): δ 8.43 (br s, 1H, NH), 7.70 (d, 1H, $J_{5,6} = 7.9$ Hz, H-6), 6.45 (d, 1H, $J_{1',2'} = 1.5$ Hz, H-1′), 6.34 (d, 1H, $J_{2',3'} = 10.1$ Hz, H-3′), 5.91 (dd, 1H, H-2′), 5.75 (d, 1H, $J_{5,6} = 7.9$ Hz, H-5), 5.29–5.26 (m, 1H, H-4′), 4.31 (dd, 1H, $J_{5',6b'} = 5.8$, $J_{6a',6b'} = 12.2$ Hz, H-6b′), 4.21 (dd, 1H, $J_{5',6a'} = 3.4$ Hz, H-6a′), 4.10 (dd, 1H, H-5′), 2.15, 2.10 (2s, 6H, 2OAc); ESI-MS (*m/z*) 325.17 [M + H]. Anal. Calcd. for $C_{14}H_{16}N_2O_7$: C, 51.85; H, 4.97; N, 8.64. Found: C, 51.98; H, 4.72; N, 8.78.

*4.1.13. 1-(2,3-Dideoxy-α-D-erythro-hex-2-enopyranosyl) uracil (**14**)*

Compound **13** (0.69 g, 2.13 mmol) was treated with ammonia/MeOH (saturated at 0 °C, 120 mL). The solution was stirred overnight at room temperature and then was concentrated under reduced pressure. Purification by flash chromatography (EtOAc/MeOH, 9:1) yielded **14** (0.33 g, 64%, $R_f = 0.4$), as a viscous oil. $[\alpha]_D^{22}$ 16.6 (*c* 0.130, MeOH); UV (MeOH): λ_{max} 261 nm (ε 6302); ^{1}H NMR (CD_3OD): δ 7.69 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 6.38 (d, 1H, $J_{2',3'} = 10.1$ Hz, H-3′), 6.30 (s, 1H, H-1′), 5.73 (d, 1H, H-2′), 5.68 (d, 1H, H-5), 4.19–4.16 (m, 1H, H-4′), 3.81 (dd, 1H, $J_{5',6a'} = 1.8$, $J_{6a',6b'} = 12.1$ Hz, H-6a′), 3.71 (dd, 1H, $J_{5',6b'} = 5.1$ Hz, H-6b′), 3.43 (dd, 1H, H-5′), ESI-MS: (*m/z*) 241.35 [M + H]. Anal. Calcd. for $C_{10}H_{12}N_2O_5$: C, 50.00; H, 5.04; N, 11.66. Found: C, 50.32; H, 5.17; N, 11.52.

*4.1.14. 1-(2,3-Dideoxy-6-O-trityl-α-D-erythro-hex-2-enopyranosyl)uracil (**15**)*

To a solution of **14** (0.33 g, 1.36 mmol) in pyridine (7 mL) were added triphenylmethyl chloride (0.49 g, 1.77 mmol) and a catalytic amount of 4-dimethylaminopyridine. The reaction mixture was stirred overnight at room temperature. After being

quenched with MeOH and concentrated, the residue was purified by flash chromatography (EtOAc/hexane, 7:3) to give **15** (0.49 g, 75%, $R_f = 0.65$ in EtOAc/MeOH, 9.5:0.5) as a yellowish solid, m.p. 185–187 °C. $[\alpha]_D^{22}$ 10.8 (*c* 0.150, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 7244); ^{1}H NMR ($CDCl_3$): δ 8.32 (br s, 1H, NH), 7.48 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 7.41–7.25 (m, 15H, $3C_6H_5$), 6.31 (dt, 1H, $J_{2',3'} = 10.2$, $J_{3',4'} = 1.8$ Hz, H-3′), 6.26 (d, 1H, $J_{1',2'} = 2.1$ Hz H-1′), 5.71–5.67 (m, 2H, H-2′, H-5), 4.21 (dd, 1H, $J_{4',5'} = 8.1$ Hz, H-4′), 3.56 (dd, 1H, $J_{5',6a'} = 4.5$, $J_{6a',6b'} = 9.5$ Hz, H-6a′), 3.47–3.42 (m, 1H, $J_{5',6b'} = 7.2$ Hz, H-5′), 3.36 (dd, 1H, H-6b′); ESI-MS (*m*/*z*) 483.69 [M + H]. Anal. Calcd. for $C_{29}H_{26}N_2O_5$: C, 72.18; H, 5.43; N, 5.81. Found: C, 72.37; H, 5.32; N, 5.97.

*4.1.15. 1-(2,3-Dideoxy-6-O-trityl-α-D-glycero-hex-2-enopyranosyl-4-ulose) uracil (**16**)*

A mixture of **15** (0.49 g, 1.02 mmol), PDC (0.46 g, 1.22 mmol) and Ac_2O (0.29 mL, 3.06 mmol) was stirred in dry CH_2Cl_2 (12 mL) for 4 h, under nitrogen at room temperature. Purification by flash chromatography (EtOAc/hexane, 6:4) yielded pure **16** (0.41 g, 83%, $R_f = 0.55$ in EtOAc/hexane, 7:3) as a white solid m.p. 110–113 °C; $[\alpha]_D^{22}$ 17.4 (*c* 0.250, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 9580); ^{1}H NMR ($CDCl_3$): δ 8.31 (br s, 1H, NH), 7.37 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 7.30–7.21 (m, 15H, $3C_6H_5$), 7.10 (t, 1H, $J_{1',2'} = J_{1',3'} = 1.5$ Hz, H-1′), 6.98 (dd, 1H, $J_{2',3'} = 10.5$ Hz, H-3′), 6.59 (dd, 1H, H-2′), 5.77 (d, 1H, $J_{5,6} = 8.1$ Hz, H-5), 4.41 (dd, 1H, $J_{5',6a'} = 2.5$, $J_{5',6b'} = 3.6$ Hz, H-5′), 3.75 (dd, 1H, $J_{6a',6b'} = 10.4$ Hz, H-6b′), 3.47 (dd, 1H, H-6a′); ESI-MS (*m*/*z*) 481.64 [M + H]. Anal. Calcd. for $C_{29}H_{24}N_2O_5$: C, 72.49; H, 5.03; N, 5.83. Found: C, 72.62; H, 5.28; N, 5.61.

*4.1.16. 1-(2,3,4-Trideoxy-4-methylene-6-O-trityl-α-D-glycero-hex-2-enopyranosyl)uracil (**10**)*

To a stirred suspension of Ph_3PCH_3Br (0.49 g, 1.37 mmol) and *t*-amyl alcohol (0.16 mL, 1.5 mmol) in dry THF (4 mL) was added sodium hydride (0.04 g, 60% in oil, 1.5 mmol) at 0 °C under nitrogen and the reaction mixture was stirred for 2 h at ambient temperature under nitrogen. To this yellow phosphorous ylide was added a solution of **16** (0.20 g, 0.42 mmol) in dry THF (0.80 mL) dropwise, at 0 °C under nitrogen. After the mixture was stirred for 30 min at ambient temperature under nitrogen, the reaction mixture was quenched with saturated sodium bicarbonate and extracted with EtOAc. The organic layer was washed with water, dried with sodium sulfate and evaporated. The residue was purified by flash chromatography (EtOAc/hexane, 5:5) to give **10** (0.13 g, 65%, $R_f = 0.4$), as a yellowish syrup.

*4.1.17. 1-(2,3-Dideoxy-α-D-glycero-hex-2-enopyranosyl-4-ulose)uracil (**17**)*

Compound **16** (0.20 g, 0.42 mmol) was dissolved in a mixture of formic acid/diethyl ether (11 mL, 1:1). The mixture was stirred for 7 min at room temperature, diluted with Tol and co-distilled several times with the same solvent to avoid ester formation [58]. The concentrated residue was purified by flash chromatography (EtOAc/hexane, 5:5) to afford pure **17** (0.08 g, 80%, $R_f = 0.25$) as a viscous oil. $[\alpha]_D^{22}$ −4.4 (*c* 0.240, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 9162); ^{1}H NMR ($CDCl_3$): δ 8.40 (br s, 1H, NH), 7.32 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 6.91 (d, 1H, $J_{2',3'} = 8.9$ Hz, H-3′), 6.88 (s, 1H, H-1′), 6.47 (d, 1H, H-2′), 5.78 (d, 1H, H-5), 4.37 (dd, 1H, $J_{5',6a'} = 2.9$, $J_{5',6b'} = 4.6$ Hz, H-5′), 4.03 (dd, 1H, $J_{6a',6b'} = 12.1$ Hz, H-6b′), 3.97 (dd, 1H, H-6a′); ESI-MS (*m*/*z*) 239.44 [M + H]. Anal. Calcd. for $C_{10}H_{10}N_2O_5$: C, 50.42; H, 4.23; N, 11.76. Found: C, 50.74; H, 4.38; N, 11.42.

*4.1.18. 1-(2,3,4-Trideoxy-4-methylene-α-D-glycero-hex-2-enopyranosyl)uracil (**18**)*

Compound **18** was synthesized from **10** (0.30 g, 0.63 mmol) by the similar procedure as described for **17** and purified by flash chromatography (EtOAc/hexane, 5:5) to afford pure **18** (0.12 g, 81%, $R_f = 0.25$), as a colorless oil. $[\alpha]_D^{22}$ −7.3 (*c* 0.140, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 5246); ^{1}H NMR ($CDCl_3$): δ 8.40 (br s, 1H, NH), 7.36 (d, 1H, $J_{5,6} = 8.1$ Hz, H-6), 6.65 (d, 1H, $J_{2',3'} = 10.1$ Hz, H-3′), 6.55 (s, 1H, H-1′), 5.74–5.71 (m, 2H, H-2′, H-5), 5.23, 5.15 (br s, 2H, methylene), 4.50 (dd, 1H, $J_{5',6a'} = 3.6$, $J_{5',6b'} = 7.5$ Hz, H-5′), 3.91 (dd, 1H, $J_{6a',6b'} = 12.1$ Hz, H-6b′), 3.80 (dd, 1H, H-6a′); ESI-MS (*m*/*z*) 237.31 [M + H]. Anal. Calcd. for $C_{11}H_{12}N_2O_4$: C, 55.93; H, 5.12; N, 11.86. Found: C, 55.69; H, 5.28; N, 11.72.

5. Molecular modelling

Computer calculations were performed on a Silicon Graphics O2 workstation using Quanta 2005 by Accelrys Inc. Grid Scan search was performed and the derived conformations were examined for their agreement with the experimental NMR data (*J* couplings). These conformations represent local minima identified at the potential energy surface.

5.1. Generating the starting conformation

The extended structures of **8**, **10**, **17** and **18** molecules were built, using standard parameters for all atoms, and then minimized with a succession of three methods [59,60]: Steepest Descents (SD) algorithm to remove unfavorable steric contacts then conjugate gradients (CONJ) to find its local minimum, followed by Powell method to reach the local minimum. CHARMm [61] force field was employed for all energy minimizations. The energy convergence criterion was root mean square (RMSD) force ≤ 0.001 kcal mol^{-1} Å^{-1}. The resulting conformations were used as initial following by systematic Grid Scan search.

5.2. Grid Scan search

The lowest energy conformations, obtained from each cluster, were further explored using systematic Grid Scan search. Intervals of 10° were applied for the single bond rotation ($C_{1'}$–N_1 and $C_{5'}$–$C_{6'}$) following by energy minimization using 2000 steps of Steepest Descents algorithm. Finally, the

lowest energy conformation for each molecule was further used in order to calculate the selected torsion angles (Table 1) and was checked for consistency with NMR J couplings.

6. Methods for measurement of biological activity

6.1. Cells and culture conditions

The human colonic adenocarcinoma Caco-2 cells were a generous gift of Dr. Rene L'Harridon, INRA, VIM, Jouy-en-Josas, France; human foetal small intestine cell line H4, breast carcinoma cell line MCF-7 and skin melanoma cell line were used. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, Grand Island, USA), supplemented with 5% foetal calf serum (Cambrex, Verviers, Belgium), L-glutamine (2 mmol/L, Sigma, St. Louis, USA), penicillin (100 U/mL, Sigma, St. Louis, USA) and streptomycin (1 mg/mL, Fluka, Buchs, Switzerland) at 37 °C in 5% carbon dioxide (CO_2) atmosphere in tissue culture flasks until confluent. Cell culture medium was regularly changed.

6.2. Unsaturated exomethylene pyranonucleosides

Stock drug solutions were freshly prepared in sterile dimethyl sulfoxide (DMSO) at the concentration of 0.5 mg/mL. The final concentration of DMSO in the cell culture medium was less than 0.1%. All solutions were protected against light.

AZT (Retrovir®) GlaxoSmithKline, USA, a drug used for antiretroviral therapy (ART) was used as a standard compound in antiviral experiments and **5FU** as a standard compound in antitumor experiments. Solutions were prepared in the same way as those of unsaturated exomethylene pyranonucleosides.

6.3. Virus propagation

Rotavirus RF strain was propagated on Caco-2 monolayers in the presence of trypsin (1 μg/mL of DMEM) as described previously [62]. Supernatant containing the virus was collected from the flasks when cytopathic effect (CPE) was observed (24–48 h at 37 °C) by microscopy and clarified by centrifugation. Virus was stored at −70 °C until used. For the antiviral assay, virus with 1.5 tissue culture infective dose 50% U/mL ($TCID_{50}$/mL) was used (100 μL per well).

6.4. Antiviral assay

The potential antiviral activity of the newly synthesized compounds was tested against rotavirus by investigating:

(i) *The inhibition of infectivity following virus attachment*: Washed monolayer Caco-2 cells were first incubated with rotavirus for 1 h at 37 °C in the presence of 5% CO_2 (time for virus to attach to cell membrane receptors). After incubation, the remaining virus was washed off with DMEM without supplements and monolayer was treated immediately with the nucleosides added in 3-fold serial dilutions (initial concentration of 0.5 mg/mL). After 72 h of incubation for rotavirus, the plates were stained with Crystal Violet in ethanol, rinsed with water, and destained with 10% (v/v) acetic acid. The A_{590} was measured, and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of absorbances in virus-infected wells ($n = 6$) compared to those in control (only virus infected) wells ($n = 6$). The minimal inhibitory concentration (IC_{50}) of the tested compounds was obtained from the concentration–effect curve.

(ii) *The neutralization of the virus in solution before attachment*: 3-fold dilutions of each tested compound (initial concentration of 0.5 mg/mL) were first pre-incubated with rotavirus in DMEM supplemented with trypsin for 12 h prior to the infection of cell monolayer at 37 °C and 5% CO_2. Residual viral infectivity was measured after 72 h post-infection for rotavirus. Rotavirus alone was treated in the same way as the control. After 72 h of incubation, the plates were stained with Crystal Violet in ethanol, rinsed with water, and destained with 10% (v/v) acetic acid. The A_{590} was measured, and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of absorbances in virus-infected wells ($n = 6$) in comparison to those in control (only virus infected) wells ($n = 6$). The minimal inhibitory concentration (IC_{50}) of the tested compounds was obtained from the concentration–effect curve.

6.5. Growth inhibition assay

It was performed on Caco-2 cell line by modified method described previously [63]. Briefly, in 96-well plates, six wells of 3-fold dilutions of each compound (initial concentration of 0.5 mg/mL) were applied to monolayers of 10 cells/well in DMEM/10% foetal bovine serum. Incubation was performed at 37 °C in the humidified incubator for 10 days. The colonies were counted in each well and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of colony number in treated wells ($n = 2$) in contrast with those in control wells ($n = 24$). The minimal inhibitory concentration (IC_{50}) of the tested compounds was obtained from the concentration–effect curve.

6.6. Cytotoxicity assay

Caco-2, H4, MCF-7, and skin melanoma cells (6×10^6 cells/plate) were seeded in P 96 plate and treated with the compounds at 3-fold serial dilutions of each compound (initial concentration of 0.5 mg/mL). Then, the cells were incubated at 37 °C in the humidified incubator for 72 h. The plates were stained with Crystal Violet in ethanol, rinsed with water, and destained with 10% (v/v) acetic acid. The A_{590} was measured, and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of absorbances in treated wells ($n = 2$) compared to those in control wells ($n = 24$). The minimal inhibitory concentration (CC_{50}) of the tested compounds was obtained from the concentration–effect curve.

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References

[1] W. Zhou, G. Gumina, Y. Chong, J. Wang, R.F. Shinazi, C.K. Chu, J. Med. Chem. 47 (2004) 3399–3408.
[2] C. Perigaud, G. Gosselin, J.L. Imbach, Nucleosides Nucleotides 11 (1992) 903–905.
[3] R.K. Robins, G.D. Kini, in: D.E.V. Wilman (Ed.), The Chemistry of Antitumor Agents, Chapman and Hall, New York, 1990, pp. 299–321.
[4] M. MacCoss, M.J. Robins, in: D.E.V. Wilman (Ed.), The Chemistry of Antitumor Agents, Chapman and Hall, New York, 1990, pp. 261–298.
[5] I. Verheggen, A. Van Aerschot, S. Toppet, R. Snoeck, G. Janssen, J. Balzarini, E. De Clercq, P. Herdewijn, J. Med. Chem. 36 (1993) 2033–2040.
[6] E. De Clercq, Biochim. Biophys. Acta 1587 (2002) 258–275.
[7] R.C. Brady, D.I. Bernstein, Antiviral Res. 61 (2004) 73–81.
[8] G.V. Papatheodoridis, E. Dimou, V. Papadimitropoulos, Am. J. Gastroenterol. 97 (2002) 1618–1628.
[9] I. Verheggen, A. Van Aerschot, L. Van Meervelt, J. Rozenski, L. Wiebe, R. Snoeck, G. Andrei, J. Balzarini, P. Claes, E. De Clercq, P. Herdewijn, J. Med. Chem. 38 (1995) 826–835.
[10] T. Ostrowski, B. Wroblowski, R. Busson, J. Rozenski, E. De Clercq, M.S. Bennet, J.N. Champness, W.C. Summers, M.R. Sanderson, P. Herdewijn, J. Med. Chem. 41 (1998) 4343–4353.
[11] Y. Maurinsh, J. Schraml, H. De Winter, N. Blaton, O. Peeters, E. Lescrinier, J. Rozenski, A. Van Aerschot, E. De Clercq, R. Busson, P. Herdewijn, J. Org. Chem. 62 (1997) 2861–2871.
[12] A. Haouz, V. Vanheusden, H. Munier-Lechman, M. Froeyen, P. Herdewijn, S. Van Galenbergh, M. Delarue, J. Biol. Chem. 278 (2003) 4963–4971.
[13] K. Vastmans, S. Pochet, A. Peys, L. Kerremans, A. Van Aerschot, C. Hendrix, P. Marliere, P. Herdewijn, Biochemistry 39 (2000) 12757–12765.
[14] K. Vastmans, M. Froeyen, L. Kerremans, S. Pochet, P. Herdewijn, Nucleic Acids Res. 29 (2001) 3154–3163.
[15] V. Nair, G.S. Buenger, J. Org. Chem. 55 (1990) 3695–3697.
[16] P. Herdewijn, E. De Clercq, Bioorg. Med. Chem. Lett. 11 (2001) 1591–1597.
[17] M.J. Ergon, F. Leclercq, K. Antonakis, I. Bennani-Baiti, C. Frayssinet, Carbohydr. Res. 248 (1993) 143–150.
[18] M. Alaoui, M.J. Egron, M. Bessodes, K. Antonakis, I. Chouroulinkov, Eur. J. Med. Chem. 22 (1987) 305–310.
[19] F. Leclercq, K. Antonakis, Carbohydr. Res. 263 (1994) 309–313.
[20] S. Manta, G. Agelis, T. Botić, A. Cencič, D. Komiotis, Bioorg. Med. Chem. 15 (2007) 980–987.
[21] S. Manta, G. Agelis, T. Botić, A. Cencič, D. Komiotis, Eur. J. Med. Chem. 43 (2008) 420–428.
[22] K. Antonakis, I. Chouroulinkov, Biochem. Pharmacol. 23 (1974) 2095–2100.
[23] T. Halmos, A. Cardon, K. Antonakis, Chem. Biol. Interact. 46 (1983) 11–29.
[24] K. Antonakis, T. Halmos, J. Bach, I. Chouroulinkov, Eur. J. Med. Chem. 15 (1980) 237–240.
[25] I. Chouroulinkov, K. Antonakis, C.R. Acad. Sci. Hebd. Seances Acad. Sci. D. 285 (1977) 1021–1024.
[26] K. Antonakis, Chimia 29 (1975) 59.
[27] K. Antonakis, M.J. Egron, Carbohydr. Res. 27 (1973) 468–470.
[28] T. Halmos, D. Komiotis, K. Antonakis, Carbohydr. Res. 145 (1985) 163–168.
[29] J. Paterson, C. Uriel, M.J. Egron, J. Herscovici, K. Antonakis, M. Alaoui, Antimicrob. Agents Chemother. 42 (1998) 779–784.
[30] M. Alaoui, C. Lasnes, K. Antonakis, I. Chouroulinkov, Mutagenesis 1 (1986) 411–417.
[31] C.H. Baker, J. Banzon, J.M. Bollinger, J. Stuble, V. Samano, M.J. Robins, B. Lippert, E. Jarvi, R. Resvick, J. Med. Chem. 34 (1991) 1879–1884.
[32] M.J. Robins, V. Samano, W. Zhang, J. Balzarini, E. De Clercq, R.T. Borchardt, Y. Lee, C.S. Yuan, J. Med. Chem. 35 (1992) 2283–2293.
[33] A.H. Cory, V. Samano, M.J. Robins, J.G. Cory, Biochem. Pharmacol. 47 (1994) 365–371.
[34] C.G. Bridges, S.P. Ahmed, P.S. Sunkara, J.R. McCarthy, A.S. Tyms, Antiviral Res. 27 (1995) 325–334.
[35] T. Takahashi, A. Nakashima, J. Kanazawa, K. Yamaguchi, S. Akinaga, T. Tamaoki, M. Okabe, Cancer Chemother. Pharmacol. 41 (1998) 268–274.
[36] G.S. Bisacchi, S.T. Chao, C. Bachard, J.P. Daris, S. Innaimo, G.A. Jacobs, O. Kocy, P. Lapointe, A. Martel, Z. Merchant, W.A. Slusarchyk, J.E. Sundeen, M.G. Young, R. Colonno, R. Zahler, Bioorg. Med. Chem. Lett. 7 (1997) 127–132.
[37] L.S. Jeong, S.J. Yoo, Bioorg. Med. Chem. Lett. 8 (1998) 847–852.
[38] S.J. Yoo, H.O. Kim, Y. Lim, J. Kim, L.S. Jeong, Bioorg. Med. Chem. 10 (2002) 215–226.
[39] P. Gunaga, M. Baba, L.S. Jeong, J. Org. Chem. 69 (2004) 3208–3211.
[40] A.E.A. Hassan, S. Shuto, A. Matsuda, Tetrahedron 50 (1994) 689–700.
[41] G. Agelis, N. Tzioumaki, T. Botić, A. Cencič, D. Komiotis, Bioorg. Med. Chem. 15 (2007) 5448–5456.
[42] J. Milecki, E. Zamaratski, T.V. Maltseva, A. Foldesi, R.W. Adamiak, J. Chattopadhyaya, Tetrahedron 55 (1999) 6603–6622.
[43] V. Vanheusden, R. Busson, P. Herdewijn, S. Van Calenbergh, J. Org. Chem. 69 (2004) 4446–4453.
[44] J. Herscovici, K. Antonakis, J. Chem. Soc., Chem. Commun. 12 (1980) 561–562.
[45] E.J. Corey, R.A.E. Winter, J. Am. Chem. Soc. 85 (1963) 2677–2678.
[46] E.J. Corey, P.B. Hopkins, Tetrahedron Lett. 23 (1982) 1979–1982.
[47] A.G.M. Barret, D.H.R. Barton, R. Bielski, J. Chem. Soc., Perkin Trans. I (1979) 2379–2381.
[48] S. Hanessian, A. Bargiotti, M. LaRue, Tetrahedron Lett. 19 (1978) 737–740.
[49] P.J. Garegg, B. Samuelsson, Synthesis (1979) 469–470.
[50] Z. Liu, B. Classon, B. Samuelsson, J. Org. Chem. 55 (1990) 4273–4275.
[51] P.J. Garegg, B. Samuelsson, Synthesis (1979) 813–814.
[52] A.F. Luzzio, E.M. Menes, J. Org. Chem. 59 (1994) 7267–7272.
[53] J. Zhang, D.L.J. Clive, J. Org. Chem. 64 (1999) 1754–1757.
[54] M. Bessodes, E. Abushanab, R.P. Panzica, J. Chem. Soc., Chem. Commun. (1981) 26–28.
[55] B.K. Chun, G.Y. Song, C.K. Chu, J. Org. Chem. 66 (2001) 4852–4858.
[56] M. Bessodes, D. Komiotis, K. Antonakis, Tetrahedron Lett. 27 (1986) 579–580.
[57] J.R. Mackey, S.J. Baldwin, J.D. Young, C.E. Cass, Drug Resist. Updates 1 (1998) 310–324.
[58] D. Komiotis, G. Agelis, S. Manta, N. Tzioumaki, E. Tsoukala, J. Carbohydr. Chem. 25 (2006) 441–450.
[59] E.D. Mantzourani, T.V. Tselios, S. Golič Grdadolnik, A. Brancale, J.A. Platts, J.M. Matsoukas, T.M. Mavromoustakos, J. Mol. Graph. Model. 25 (2005) 17–29.
[60] E.D. Mantzourani, T.V. Tselios, S. Golič Grdadolnik, J.A. Platts, A. Brancale, G. Deraos, J.M. Matsoukas, T.M. Mavromoustakos, J. Med. Chem. 49 (2006) 6683–6691.
[61] B.R. Brooks, R.E. Bruccoleri, B.D. Olafson, D.J. States, S. Swaminathan, M. Karplus, J. Comput. Chem. 4 (1983) 187–217.
[62] S. Kitaoka, H. Suzuki, Y. Numazaki, T. Konno, N. Ishida, Tohoku J. Exp. Med. 149 (1986) 437–447.
[63] A. Cencič, C. Henry, F. Lefevre, J.H. Huet, S. Koren, C. La Bonnardiere, Eur. J. Biochem. 269 (2002) 2772–2781.