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Short communication

Unsaturated fluoro-ketopyranosyl nucleosides: Synthesis and biological evaluation of 3-fluoro-4-keto-β-D-glucopyranosyl derivatives of N^4-benzoyl cytosine and N^6-benzoyl adenine

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Abstract

The protected β-nucleosides 1-(2,4,6-tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**2a**) and 9-(2,4,6-tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^6-benzoyl adenine (**2b**), were synthesized by the coupling of peracetylated 3-deoxy-3-fluoro-D-glucopyranose (**1**) with silylated N^4-benzoyl cytosine and N^6-benzoyl adenine, respectively. The nucleosides were deacetylated and several subsequent protection and deprotection steps afforded the partially acetylated nucleosides of cytosine **7a** and adenine **7b**, respectively. Finally, direct oxidation of the free hydroxyl group at 4′-position of **7a** and **7b**, and simultaneous elimination reaction of the β-acetoxyl group, afforded the desired unsaturated 3-fluoro-4-keto-β-D-glucopyranosyl derivatives. These newly synthesized compounds were evaluated for their potential antitumor and antiviral activities. Compared to 5FU, the newly synthesized derivatives showed to be more efficient as antitumor growth inhibitors and they exhibited direct antiviral effect toward rotavirus.

Keywords: Unsaturated fluoro ketonucleosides; β-Elimination reaction; Antiviral; Antitumor activity

1. Introduction

Nucleoside analogues have been widely explored as potential antiviral and antitumoral chemotherapeutic agents [1–4]. Consequently, a large number of modifications have been made to both base and sugar moieties of natural nucleosides, with the objective of increasing the therapeutic index of established antiviral agents [5]. Over the past two decades, nucleoside chemistry has evolved to facilitate efficient routes to effective agents for the treatment of AIDS [6], herpes [7] and viral hepatitis [8].

The last decades, nucleosides with a six-membered carbohydrate moiety have been evaluated for their potential antiviral [9–12] and antibiotic [13] properties and as building blocks in nucleic acid synthesis [14,15]. Removal of the hydroxyl groups in the 2′- and 3′-positions of the aforementioned nucleosides has generated drugs of choice for treatment of certain viral infections, including human immunodeficiency virus (HIV) infection [16]. Furthermore, replacement of the glycane moiety with a pseudo sugar, a substituted cyclohexene ring, furnished enzymatically stable nucleic acid analogues [17] which can be considered as biosteres of natural nucleosides and nucleotides, and can play a major role in different domains like therapy, diagnosis, and biotechnology. One series of uncommon six-membered nucleoside analogues, the unsaturated ketonucleosides, are well established for their antineoplastic activity and immunosuppressive effects [18–20]. It appeared that these nucleosides not only exhibit growth inhibitory activity against a variety of tumor cells [21,22] *in vitro* and L1210 leukemia [23,24] *in vivo*, but they also may constitute

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important synthetic intermediates in the nucleoside field owing to their chemical reactivities in various media [25,26].

On the other hand, fluorine substitution has been extensively investigated as a means of enhancing biological activity and increasing chemical or metabolic stability [27–36]. It was proved that the introduction of a fluorine atom in the sugar moiety of the unsaturated nucleosides increases the activity [37], raises the lipophilicity, and makes the penetration of the drug through the cell membrane easier [38–41]. Specific fluorination at the 2′- and/or 3′-position of the sugar moiety of the nucleoside analogues has been studied in the pursuit of safe, effective and chemically stable antiviral agents [38,42–50].

In recent years a number of cytosine and adenine nucleoside analogues have been widely used not only as antileukemic agents, but also as antitumor agents against solid tumors [51–63]. Moreover, cytosine unsaturated nucleoside analogues, such as D-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (D-d4FC) and its L-enantiomer (L-Fd4C), have been identified as *anti*-HIV agents [64,65] and structure–activity relationships of 3′-fluoro-2′,3′-unsaturated nucleosides, showed potent activity in the cytosine derivatives [66].

As a part of our continuing effort to develop new tumor-inhibitory agents, unsaturated 3′-fluoro-2′-ketonucleosides of N^4-benzoyl cytosine have been prepared and showed to have a promising potential in combating the rotaviral infections and in the treatment of colon cancer [67]. In extending these studies, we decided to design and synthesize a new class of unsaturated 3′-fluoro-4′-ketonucleosides, that of N^4-benzoyl cytosine and N^6-benzoyl adenine, respectively.

2. Results and discussion

2.1. Chemistry

The starting materials, 1-(2,4,6-tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**2a**) and 9-(2,4,6-tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^6-benzoyl adenine (**2b**), were obtained from condensation of 1,2,4,6-tetra-*O*-acetyl-3-deoxy-3-fluoro-glucopyranose (**1**) [68,69] with silylated N^4-benzoyl cytosine and N^6-benzoyl adenine, respectively, in the presence of trimethylsilyl trifluomethane-sulfonate and tin chloride, in refluxing acetonitrile. The coupling of the peracetylated sugar **1** with the silylated N^4-benzoyl cytosine and N^6-benzoyl adenine, yielded only the β-nucleoside derivatives **2a** and **2b** (68 and 60%) after standard workup and flash chromatography. The β configuration of these nucleosides was clearly established by their ^{1}H NMR spectra. Selective deprotection of **2a** and **2b** using NaOH–ethanol–pyridine [70] gave the base protected benzoylated derivatives **3a** and **3b** in excellent yield (90 and 88%, respectively). It is noteworthy that the fully unprotected derivatives were obtained when the β protected nucleosides were treated by methanolic ammonia [71], or potassium carbonate–methanol [72], or guanidine [73]. Treatment of **3a** and **3b** with 2,2-dimethoxypropane in dry *N*,*N*-dimethylformamide gave the 4′,6′-isopropylidene derivatives **4a** and **4b**, in 80 and 76% yield, respectively (Scheme 1).

Acetylation of the free hydroxyl group in the 2′-position of the sugar moiety of both the nucleosides with acetic anhydride/pyridine afforded the desired acetylated derivatives **5a** and **5b** in very good yield (89 and 80%). The 4′,6′-*O*-isopropylidene group of both the derivatives was removed upon short treatment with 90% trifluoroacetic acid in methanol. Selective protection of the primary 6′-hydroxyl group with a trityl group yielded **7a** and **7b** (60 and 62%, respectively). Oxidation of the fluoro acetylated precursors **7a** and **7b**, was performed with pyridinium dichromate (PDC)/acetic anhydride and afforded, after a β-elimination reaction, the desired, unsaturated carbonyl compounds 1-(3-deoxy-3-fluoro-6-*O*-trityl-β-D-*glycero*-hex-2-enopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**) in 75% yield and 9-(3-deoxy-3-fluoro-6-*O*-trityl-β-D-*glycero*-hex-2-enopyranosyl-4-ulose)-N^6-benzoyl adenine (**8b**) in 50% yield.

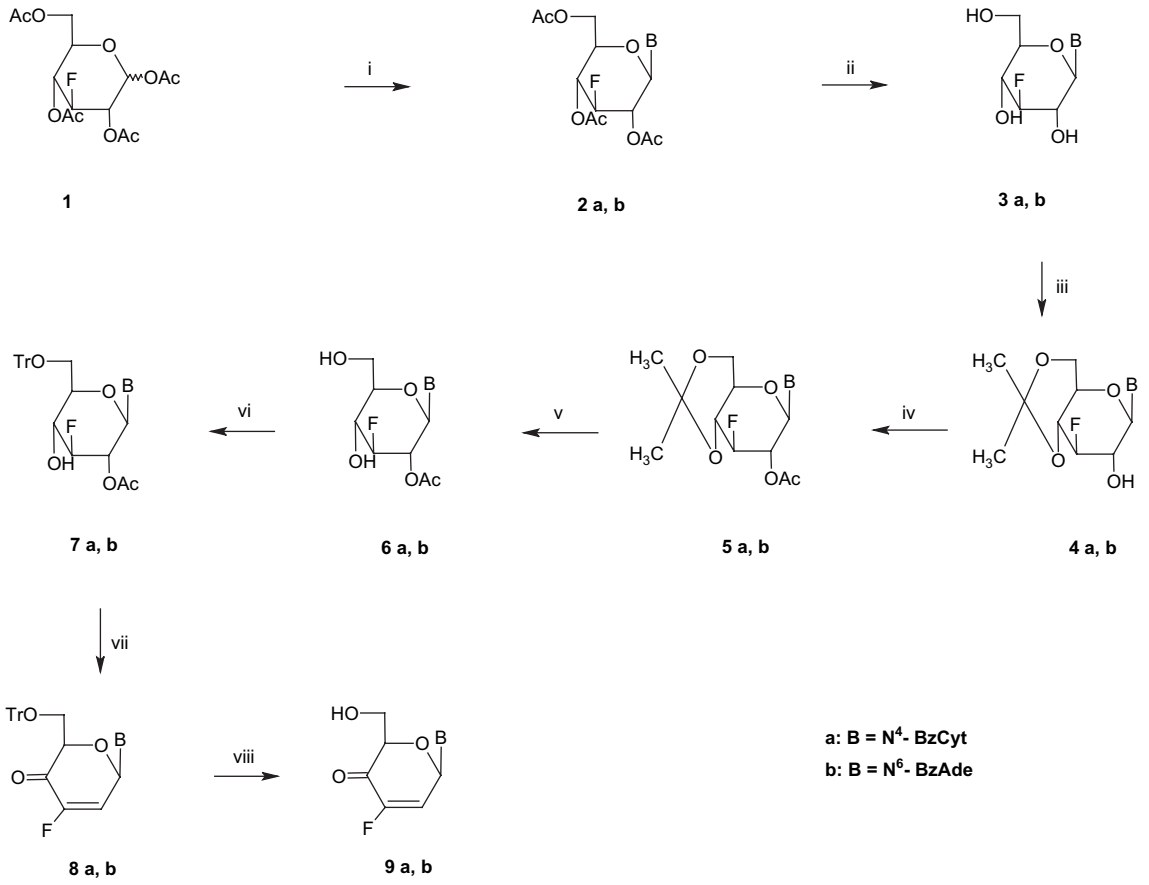
Finally, detritylation of **8a** using a mixture of formic acid and diethyl ether [74], afforded the corresponding deprotected unsaturated 3′-fluoro-4′-keto nucleoside of N^4-benzoyl cytosine **9a** in 50% yield.

Several approaches to obtain the desired detritylated derivative of N^6-benzoyl adenine **9b** were attempted. Treatment of **8b** with a mixture of formic acid and diethyl ether [74], or methanolic hydrogen chloride [75], or 70% acetic acid at 60 °C [76], or sodium–ammonia [77], or zinc bromide in dichloromethane [78], unfortunately, did not afford the corresponding deprotected derivative of adenine **9b** and only adenine was isolated from the reaction mixture.

All compounds were characterized by their elemental analyses, ^{1}H NMR and ESI-MS. ^{1}H NMR data for 1-(2,4,6-tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**2a**) ($J_{1'2'} = 9.4$ Hz, $J_{2'3'} = 9.1$ Hz, $J_{3'4'} = 9.0$ Hz) and 9-(2,4,6-tri-*O*-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^6-benzoyl adenine (**2b**) ($J_{1'2'} = 9.6$ Hz, $J_{2'3'} = 9.1$ Hz, $J_{3'4'} = 9.0$ Hz) showed that, as expected, these compounds had the β configuration and that it existed in the $^4C_1$ conformation. Furthermore, in compounds **8a**, **8b** and **9a**, the presence of the C(O)–CH=CH-system was ascertained by the disappearance, in the ^{1}H NMR spectrum, of the signal for H-4′ and the deshielding of other protons, especially the allylic proton H-2′ as previously observed for other (2-deoxy-β-D-hex-2-enopyranosyl-4-ulose) nucleosides [79–81]. Moreover, the small value of $J_{1'2'}$ (1.6 Hz for **8a** and 1.4 Hz for **8b**) suggests that these compounds adopt the sofa conformation [80] with H-1′ perpendicular to the ring. Such a conformation should be favoured, because the bulky substituents at C-1′ and C-5′ are equatorial.

2.2. Antiviral activity

To examine the potential antiviral properties of the nucleosides, a colon adenocarcinoma Caco-2 cell line infected with gastrointestinal rotavirus as a model virus was used. The results obtained by performing antiviral assay with the newly synthesized compounds are summarized in Table 1 and compared to AZT. Although compounds did not show antiviral activity in neutralization assay they all showed the ability to inhibit rotavirus infectivity following virus attachment. In



Scheme 1. (i) Silylated base, CH_3CN, trimethylsilyl trifluomethane-sulfonate or tin chloride; (ii) ethanol, pyridine, NaOH, 0 °C, 30 min, Amberlite IR-120 (H^+) resin; (iii) 2,2-dimethoxypropane, *p*-toluenesulfonic acid, DMF, 60 °C, 1 h; (iv) pyridine, acetic anhydride, 4 °C, 12 h; (v) 90% trifluoroacetic acid in methanol, 20 °C, 10 min; (vi) pyridine, triphenylmethyl chloride, 4-dimethylaminopyridine, 60 °C, 12 h; (vii) PDC, acetic anhydride, CH_2Cl_2–DMF, 90 °C, 100 min; (viii) formic acid, diethyl ether, 20 °C, 10 min.

comparison to AZT higher concentrations were needed for the new compounds to obtain the same effect against virus and CC_{50}/IC_{50} values were lesser for the new compounds than for AZT.

2.3. Cytotoxic and growth inhibition activity

Compounds **8a**, **8b** and **9a** were evaluated for their cytotoxic activity against several malignant tumor cell lines: colon carcinoma (Caco-2), breast carcinoma (MCF-7), skin melanoma and normal human intestinal cells (H4, control cell line). Results are summarized in Table 2. From the tested unsaturated fluoro-ketopyranosyl nucleoside analogues, 1-(3-deoxy-3-fluoro-6-*O*-trityl-β-D-*glycero*-hex-2-enopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**) exhibited the best cytotoxic effect, particularly against skin melanoma cells (CC_{50} = 3.3 μM). The same analogue of adenine **8b** showed about 3-fold less cytotoxicity toward skin melanoma cells than **8a**, and toward other malignant cell lines their activity and selectivity were comparable. In comparison to 5FU, compound **8a** showed to be 16-fold more selective in MCF-7 cells, 3-fold more selective in skin melanoma cells and 2.5-fold more selective in Caco-2 cells (see TSI values).

Generally, all tested compounds exhibit higher cytotoxicity in tumor cells than in the normal H4 cell line. Furthermore in comparison to 5FU, compounds showed to be more cytotoxic against all tumorgenic cells and more selective toward Caco-2 and MCF-7 cells. From the results obtained it can be observed that some compounds have selective effect toward specific tumor cell line used and it has been reported before that mode of inhibitory action especially on the target enzymes in carcinogenic cells is not always similar even among nucleoside antimetabolites which have the same nucleoside base [82].

Table 1
Antiviral activity of nucleosides **8a**, **8b**, **9a** 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	
8a	n.e.	n.e.	–	0.2	332.43	0.10
8b	n.e.	n.e.	–	0.5	799.17	0.04
9a	n.e.	n.e.	–	0.5	1391.56	0.04
AZT	0.02	74.84	0.75[c]	0.006	22.45	2.50

n.e. = No effect.
[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 2.
[c] CC_{50} for AZT on Caco-2 cells = 56.1 μM.

Table 2
Cytotoxic effect (CC_{50}, μM) of compounds **8a**, **8b**, **9a** and 5-fluorouracil (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
8a	831.1	33.2	3.3	9.9	25.0	251.8	83.9	1.1
8b	799.1	31.9	9.5	9.5	25.0	84.1	84.1	1.1
9a	1391.5	55.6	16.6	55.6	25.0	83.8	25.0	16.6
5FU	3843.8	384.4	46.1	768.8	10	83.4	5	1.5

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

The growth inhibition of Caco-2 cells induced by the new compounds was additionally measured by determining the minimal inhibitory concentration, IC_{50}. The results are summarized at the end of Table 2 and compared with the values obtained with 5FU. Compounds **8a** and **8b** exhibit slightly better growth inhibitory activity (IC_{50} 1.1 μM) than 5FU (IC_{50} 1.5 μM) but with higher tumor selectivity (see TSI values). Compound **9a** was also able to inhibit cell growth although the effect was somewhat less pronounced than that of 5FU but with higher selectivity for Caco-2 cells.

3. Conclusion

In conclusion to biological assays, compounds **8a**, **8b** and **9a** were evaluated for their antiviral and antitumor cell activities. Among them 1-(3-deoxy-3-fluoro-6-*O*-trityl-β-D-*glycero*-hex-2-enopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**) exhibited the best cytotoxic effect, particularly against skin melanoma cells ($CC_{50} = 3.3$μM). In general, all tested compounds had better cytotoxic activities than the corresponding standard 5FU. The compounds also showed rather modest but direct activity against rotavirus.

4. Experimental part

4.1. Chemistry

Melting points were recorded in a Mel-Temp apparatus and are uncorrected. 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 sulfuric acid. Flash chromatography was performed using silica gel (240–400 mesh, Merck).

NMR spectra were recorded at room temperature with a Brucker 400 MHz spectrometer using $CDCl_3$, CD_3OD, with internal tetramethylsilane (TMS) for ^1H and internal trifluorotoluene (TFT) for ^{19}F. The chemical shifts are expressed in parts per million (δ) and following abbreviations were used: s = singlet, br s = broad singlet, d = doublet, dd = doublet doublet, dtr = doublet triplet and m = multiplet. Mass spectra were obtained with a Micromass Platform LC (ESI-MS). All reactions were carried out in dry solvents. Dichloromethane was distilled from phosphorus pentoxide and stored over 4E molecular sieves. Acetonitrile was distilled from calcium hydride and stored over 3E molecular sieves. Dimethylformamide (DMF) was also stored over 3E molecular sieves. Pyridine was stored over pellets of potassium hydroxide.

4.2. Synthesis of 1-(3-deoxy-3-fluoro-β-D-glycero-hex-2-enopyranosyl-4-ulose)-N^4-benzoyl cytosine (**9a**)

4.2.1. Synthesis of 1-(2,4,6-tri-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**2a**)

A mixture of N^4-benzoyl cytosine (3.99 g, 18.55 mmol), hexamethyldisilazane (HMDS) (4.8 mL, 23 mmol) and saccharine (0.16 g, 0.85 mmol) in anhydrous CH_3CN (69 mL) was refluxed for 30 min at 120 °C. To this were added tetraacetylated 3-deoxy-3-fluoro-D-glucose [68,69] (**1**) (5.00 g, 14.27 mmol) and trimethylsilyl trifluomethane-sulfonate (3.6 mL, 19.98 mmol). The reaction mixture was refluxed at 120 °C for 5 h, then cooled, neutralized with aqueous sodium bicarbonate, and extracted with CH_2Cl_2 (1000 mL). The organic layer was washed with water (3 × 20 mL) and dried over anhydrous sodium sulfate, evaporated to dryness, finally purified with flash chromatography using ethyl acetate–*n*-hexane (8:2) as eluent to give compound **2a** as a solid. Yield: 4.90 g (68%), $R_f = 0.35$, m.p. 277–279 °C; ^1H NMR ($CDCl_3$): δ 7.88 (d, 1H, $J_{6,5} = 7.2$ Hz, H-6), 7.84–7.48 (m, 6H, Bz and H-5), 6.10 (d, 1H, $J_{1',2'} = 9.4$ Hz, H-1′), 5.39–5.22 (m, 2H, $J_{2',1'} = 9.4$ Hz, $J_{4',3'} = 9.0$ Hz, H-2′ and H-4′), 4.80 (dtr, 1H, $J_{F,3'} = 51.7$ Hz, $J_{3',2'} = 9.1$ Hz, $J_{3',4'} = 9.0$ Hz, H-3′), 4.34–4.11 (m, 2H, H-6a′,6b′), 3.86 (m, 1H, H-5′), 2.16 and 2.11 and 2.07 (3s, 9H, 3OAc); ^{19}F NMR: δ −65.0. Anal. calcd. for $C_{23}H_{24}FN_3O_9$: C, 54.65, H, 4.79, F, 3.76, N, 8.31; found: C, 54.70, H, 4.80, F, 3.75, N, 8.42; ESI-MS (*m*/*z*): found 506.43 ($M + H^+$).

4.2.2. Synthesis of 1-(3,4-dideoxy-3-fluoro-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**3a**)

Compound **2a** (4.90 g, 9.7 mmol) was dissolved in ethanol–pyridine (97 mL + 29.1 mL), 2 M NaOH (19.4 mL) was added and the mixture was stirred for 30 min at 0 °C. Amberlite IR-120 (H^+) was added to neutralize the base. The suspension was filtered, the resin was washed with ethanol and pyridine (100 mL + 100 mL) and the filtrate was evaporated. The solid residue was triturated with diethyl ether (2 × 30 mL) and CH_2Cl_2 (2 × 30 mL) and filtered. Crude **3a** was obtained as a yellow foam and it was used without further purification. Yield: 3.31 g (90%); ESI-MS (*m*/*z*): found 380.37 ($M + H^+$).

4.2.3. Synthesis of 1-(3-deoxy-3-fluoro-4,6-O-isopropylidene-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**4a**)

Compound **3a** (3.31 g, 8.73 mmol) was dissolved in a mixture of 34.92 mL of 2,2-dimethoxypropane and 110.4 mL of dry DMF. To this was added *p*-toluenesulfonic acid (2.65 g, 13.97 mmol) and the mixture was stirred at 60 °C for 1 h.

The reaction mixture was neutralized with triethylamine so pH did not exceed 7. The mixture was concentrated under high vacuum to eliminate the DMF. Purification with flash chromatography using ethyl acetate–*n*-hexane (9:1) gave **4a** as a bright yellow oil. Yield: 2.93 g (80%), $R_f = 0.3$; ^{1}H NMR ($CDCl_3$): δ 7.88 (d, 1H, $J_{6,5} = 7.9$ Hz, H-6), 7.84–7.43 (m, 6H, Bz and H-5), 5.86 (d, 1H, $J_{1',2'} = 9.2$ Hz, H-1′), 4.68 (dtr, 1H, $J_{F,3'} = 53.2$ Hz, $J_{3',2'} = 8.7$ Hz, $J_{3',4'} = 8.7$ Hz, H-3′), 4.05–3.87 (m, 2H, $J_{2',1'} = 9.2$ Hz, $J_{4',3'} = 8.7$ Hz, H-2′ and H-4′), 3.86–3.73 (m, 2H, H-6a′,6b′), 3.51 (m, 1H, H-5′), 1.53 and 1.45 (2s, 6H, $2 \times CH_3$); ^{19}F NMR: δ −64.9. Anal. calcd. for $C_{20}H_{22}FN_3O_6$: C, 57.28, H, 5.29, F, 4.53, N, 10.02; found: C, 57.16, H, 5.31, F, 4.24, N, 10.03; ESI-MS (*m*/*z*): found 420.41 ($M + H^+$).

4.2.4. Synthesis of 1-(2-O-acetyl-3-deoxy-3-fluoro-4,6-O-isopropylidene-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**5a**)

To a solution of **4a** (2.93 g, 6.99 mmol) in dry pyridine (35 mL) was added acetic anhydride (0.59 mL, 6.29 mmol) at 0 °C and the resulted mixture was stirred overnight at 4 °C. Methanol (0.3 mL) was added to quench the reaction and the mixture was concentrated under high vacuum to remove the solvents. Purification with flash chromatography using ethyl acetate–*n*-hexane (7:3) gave **5a**. Yield: 2.87 g (89%), $R_f = 0.4$; ^{1}H NMR ($CDCl_3$): δ 7.94 (d, 1H, $J_{6,5} = 6.5$ Hz, H-6), 7.80–7.50 (m, 6H, Bz and H-5), 6.09 (d, 1H, $J_{1',2'} = 9.3$ Hz, H-1′), 5.26 (m, 1H, H-2′), 4.76 (dtr, 1H, $J_{F,3'} = 53.0$ Hz, $J_{3',2'} = 8.9$ Hz, $J_{3',4'} = 8.8$ Hz, H-3′), 4.03 (m, 1H, H-4′), 3.99–3.83 (m, 2H, H-6a′,6b′), 3.57 (m, 1H, H-5′), 2.09 (1s, 3H, OAc), 1.59 and 1.51 (2s, 6H, $2 \times CH_3$); ^{19}F NMR: δ −65.0. Anal. calcd. for $C_{22}H_{24}FN_3O_7$: C, 57.26, H, 5.24, F, 4.12, N, 9.11; found: C, 57.14, H, 5.37, F, 4.02, N, 9.01; ESI-MS (*m*/*z*): found 462.42 ($M + H^+$).

4.2.5. Synthesis of 1-(2-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**6a**)

Product **5a** (2.87 g, 6.22 mmol), obtained from the previous procedure, was dissolved in 31.1 mL of 90% trifluoroacetic acid in methanol. The solution was stirred for 10 min at room temperature and then concentrated at 40 °C under high vacuum in order to remove traces of trifluoroacetic acid. Purification with flash chromatography using ethyl acetate gave **6a**. Yield: 2.36 g (90%), $R_f = 0.12$; ESI-MS (*m*/*z*): found 422.39 ($M + H^+$).

4.2.6. Synthesis of 1-(2-O-acetyl-3-deoxy-3-fluoro-6-O-trityl-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**7a**)

To a solution of compound **6a** (2.36 g, 5.6 mmol) in dry pyridine (28 mL) was added triphenylmethyl chloride (1.87 g, 6.72 mmol) and a catalytic amount of 4-dimethylaminopyridine. The mixture was stirred overnight at 60 °C and then concentrated. Purification with flash chromatography using ethyl acetate–*n*-hexane (7:3), gave pure **7a** as a white solid. Yield: 2.23 g (60%), $R_f = 0.37$, m.p. 184–186 °C; ^{1}H NMR ($CDCl_3$): δ 7.90 (d, 1H, $J_{6,5} = 7.5$ Hz, H-6), 7.85–7.26 (m, 21H, Bz and H-5 and $3C_6H_5$), 6.03 (d, 1H, $J_{1',2'} = 9.4$ Hz, H-1′), 5.20 (m, 1H, H-2′), 4.70 (dtr, 1H, $J_{F,3'} = 52.0$ Hz, $J_{3',2'} = 8.9$ Hz, $J_{3',4'} = 8.9$ Hz, H-3′), 4.13 (m, 1H, H-4′), 4.09–3.64 (m, 2H, H-6a′,6b′), 3.48 (m, 1H, H-5′), 2.05 (1s, 3H, OAc); ^{19}F NMR: δ −64.9. Anal. calcd. for $C_{38}H_{34}FN_3O_7$: C, 68.77, H, 5.16, F, 2.86, N 6.33; found: C, 68.56, H, 5.27, F, 2.72, N, 6.40; ESI-MS (*m*/*z*): found 664.67 ($M + H^+$).

4.2.7. Synthesis of 1-(3-deoxy-3-fluoro-6-O-trityl-β-D-glycero-hex-2-enopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**)

A mixture of **7a** (2.23 g, 3.36 mmol, dried by co-evaporation with toluene), PDC (1.9 g, 5.04 mmol) and acetic anhydride (3.17 mL, 33.60 mmol) was refluxed at 90 °C in dry CH_2Cl_2 (32 mL) and dry DMF (10 mL) for 100 min. The mixture was then concentrated under high vacuum to remove the solvents. Purification with flash chromatography using ethyl acetate–*n*-hexane (7:3) afforded pure **8a** as a white foam. Yield: 1.51 g (75%), $R_f = 0.39$; ^{1}H NMR ($CDCl_3$): δ 7.95 (d, 1H, $J_{6,5} = 7.5$ Hz, H-6), 7.90–7.24 (m, 21H, Bz and H-5 and $3C_6H_5$), 7.00 (dtr, 1H, $J_{2',1'} = 1.6$ Hz, $J_{2',5'} = 1.8$ Hz, $J_{F,2'} = 7.3$ Hz, H-2′), 6.58 (dd, 1H, $J_{1',2'} = 1.6$ Hz, $J_{F,1'} = 10.5$ Hz, H-1′), 4.50 (m, 1H, H-5′), 3.76–3.62 (m, 2H, H-6a′,6b′); ^{19}F NMR: δ −63.2. Anal. calcd. for $C_{36}H_{28}FN_3O_5$: C, 71.87, H, 4.69, F, 3.16, N, 6.98; found: C, 71.75, H, 4.88, F, 3.05, N, 7.07; ESI-MS (*m*/*z*): found 602.63 ($M + H^+$).

4.2.8. Synthesis of 1-(3-deoxy-3-fluoro-β-D-glycero-hex-2-enopyranosyl-4-ulose)-N^4-benzoyl cytosine (**9a**)

Compound **8a** (1.51 g, 2.51 mmol) was dissolved in a mixture of formic acid and diethyl ether (10 mL + 10 mL). The mixture was stirred for 10 min at room temperature, diluted with toluene and co-distilled several times with the same solvent to avoid ester formation [83]. The mixture was concentrated under high vacuum and then purified with flash chromatography using ethyl acetate to afford pure **9a**. Yield: 0.45 g (50%), $R_f = 0.35$; ^{1}H NMR (CD_3OD): δ 8.23 (d, 1H, $J_{6,5} = 7.5$ Hz, H-6), 8.04–7.38 (m, 6H, Bz and H-5), 7.08 (dtr, 1H, $J_{2',1'} = 1.4$ Hz, $J_{2',5'} = 1.9$ Hz, $J_{F,2'} = 7.0$ Hz, H-2′), 6.85 (dd, 1H, $J_{1',2'} = 1.4$ Hz, $J_{F,1'} = 11.7$ Hz, H-1′), 4.64 (m, 1H, H-5′), 4.14–3.97 (m, 2H, H-6a′,6b′); ^{19}F NMR: δ −65.0. Anal. calcd. for $C_{17}H_{14}FN_3O_5$: C, 56.83, H, 3.93, F, 5.29, N, 11.69; found: C, 56.74, H, 4.05, F, 5.14, N, 11.89; ESI-MS (*m*/*z*): found 360.29 ($M + H^+$).

4.3. Synthesis of 9-(3-deoxy-3-fluoro-6-O-trityl-β-D-glycero-hex-2-enopyranosyl-4-ulose)-N^6-benzoyl adenine (**8b**)

4.3.1. Synthesis of 9-(2,4,6-tri-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^6-benzoyl adenine (**2b**)

A mixture of N^6-benzoyl adenine (4.44 g, 18.55 mmol), HMDS (4.8 mL, 23 mmol) and saccharine (0.16 g, 0.85 mmol)

in anhydrous CH_3CN (69 mL) was refluxed for 30 min at 120 °C. To this were added tetraacetylated 3-deoxy-3-fluoro-D-glucose [68,69] (**1**) (5.00 g, 14.27 mmol) and tin chloride (2.35 mL, 19.98 mmol). The reaction mixture was refluxed at 100 °C for 5 h, then cooled, neutralized with aqueous sodium bicarbonate, and extracted with CH_2Cl_2 (1000 mL). The organic layer was washed with water (3 × 20 mL) and dried over anhydrous sodium sulfate, evaporated to dryness, finally purified with flash chromatography using ethyl acetate–*n*-hexane (8:2) as eluent to give compound **2b** as a solid. Yield: 4.53 g (60%), $R_f = 0.24$, m.p. 118–120 °C; ^{1}H NMR ($CDCl_3$): δ 9.07 (1H, br s, –NH), 8.77 and 8.19 (2H, 2s, H-2,8), 7.98–7.42 (m, 5H, Bz), 5.84 (d, 1H, $J_{1',2'} = 9.6$ Hz, H-1′), 5.70–5.58 (m, 1H, H-2′), 5.43–5.30 (m, 1H, H-4′), 4.80 (dtr, 1H, $J_{F,3'} = 51.6$ Hz, $J_{3',2'} = 9.1$ Hz, $J_{3',4'} = 9.0$ Hz), 4.29–4.08 (m, 2H, H-6a′,6b′), 3.90 (m, 1H, H-5′), 2.11, 2.01 and 1.78 (3s, 9H, 3OAc); ^{19}F NMR: δ –65.0. Anal. calcd. for $C_{24}H_{24}FN_5O_8$: C, 54.44, H, 4.57, F, 3.59, N, 13.23; found: C, 54.58, H, 4.38, F, 3.72, N, 13.39; ESI-MS (*m/z*): found 530.46 ($M + H^+$).

4.3.2. Synthesis of 9-(3,4-dideoxy-3-fluoro-β-D-glucopyranosyl)-N^6-benzoyl adenine (3b)

Adenine derivative **3b** was synthesized from **2b** by the similar procedure as described for **3a**. Yield: 3.04 g (88%), obtained as a yellow foam and it was used without further purification. ESI-MS (*m/z*): found 404.38 ($M + H^+$).

4.3.3. Synthesis of 9-(3-deoxy-3-fluoro-4,6-O-isopropylidene-β-D-glucopyranosyl)-N^6-benzoyl adenine (4b)

Adenine derivative **4b** was synthesized from **3b** by the similar procedure as described for **4a**. Purified with flash chromatography using ethyl acetate as eluent and it was obtained as a bright yellow oil. Yield: 2.54 g (76%), $R_f = 0.43$; ^{1}H NMR ($CDCl_3$): δ 9.04 (1H, br s, –NH), 8.39 and 8.0 (2H, 2s, H-2,8), 7.94–7.42 (m, 5H, Bz), 5.65 (d, 1H, $J_{1',2'} = 8.9$ Hz, H-1′), 4.70 (dtr, 1H, $J_{F,3'} = 39.5$ Hz, $J_{3',2'} = 8.9$ Hz, $J_{3',4'} = 8.7$ Hz, H-3′), 4.56 (m, 1H, H-2′), 4.10–3.94 (m, 1H, H-4′), 3.93–3.70 (m, 2H, H-6a′,6b′), 3.51 (m, 1H, H-5′), 1.52 and 1.43 (2s, 6H, 2 × CH_3); ^{19}F NMR: δ –64.9. Anal. calcd. for $C_{21}H_{22}FN_5O_5$: C, 56.88, H, 5.00, F, 4.28, N, 15.79; found: C, 56.76, H, 4.82, F, 4.42, N, 15.90; ESI-MS (*m/z*): found 444.41 ($M + H^+$).

4.3.4. Synthesis of 9-(2-O-acetyl-3-deoxy-3-fluoro-4,6-O-isopropylidene-β-D-glucopyranosyl)-N^6-benzoyl adenine (5b)

Adenine derivative **5b** was synthesized from **4b** by the similar procedure as described for **5a**. Purified with flash chromatography using ethyl acetate–*n*-hexane (6:4) as eluent and it was obtained as a yellow foam. Yield: 2.22 g (80%), $R_f = 0.2$; ^{1}H NMR ($CDCl_3$): δ 9.00 (1H, br s, –NH), 8.85 and 8.22 (2H, 2s, H-2,8), 8.06–7.52 (m, 5H, Bz), 5.91 (d, 1H, $J_{1',2'} = 9.5$ Hz, H-1′), 5.72 (m, 1H, H-2′), 4.80 (dtr, 1H, $J_{F,3'} = 52.9$ Hz, $J_{3',2'} = 8.8$ Hz, $J_{3',4'} = 8.9$ Hz, H-3′), 4.12 (m, 1H, H-4′), 4.06–3.84 (m, 2H, H-6a′,6b′), 3.64 (m, 1H, H-5′), 1.87 (1s, 3H, OAc), 1.61 and 1.52 (2s, 6H, 2 × CH_3); ^{19}F NMR: δ –65.0. Anal. calcd. for $C_{23}H_{24}FN_5O_6$: C, 56.90, H, 4.98, F, 3.91, N, 14.43; found: C, 57.03, H, 4.86, F, 4.04, N, 14.22; ESI-MS (*m/z*): found 486.47 ($M + H^+$).

4.3.5. Synthesis of 9-(2-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranosyl)-N^6-benzoyl adenine (6b)

Adenine derivative **6b** was synthesized from **5b** by the similar procedure as described for **6a**. Purified with flash chromatography using ethyl acetate as eluent and it was obtained as a white foam. Yield: 1.83 g (90%), $R_f = 0.1$; ESI-MS (*m/z*): found 446.38 ($M + H^+$).

4.3.6. Synthesis of 9-(2-O-acetyl-3-deoxy-3-fluoro-6-O-trityl-β-D-glucopyranosyl)-N^6-benzoyl adenine (7b)

Adenine derivative **7b** was synthesized from **6b** by the similar procedure as described for **7a**. Purified with flash chromatography using ethyl acetate–*n*-hexane (7:3) as eluent and it was obtained as a white solid. Yield: 1.75 g (62%), $R_f = 0.43$, m.p. 147–149 °C; ^{1}H NMR ($CDCl_3$): δ 9.20 (1H, br s, –NH), 8.87 and 8.28 (2H, 2s, H-2,8), 8.10–7.22 (m, 20H, Bz and 3C_6H_5), 5.88 (d, 1H, $J_{1',2'} = 9.5$ Hz, H-1′), 5.61 (m, 1H, H-2′), 4.73 (dtr, 1H, $J_{F,3'} = 52.1$ Hz, $J_{3',2'} = 8.9$ Hz, $J_{3',4'} = 8.9$ Hz, H-3′), 4.21 (m, 1H, H-4′), 3.75 (m, 1H, H-5′), 3.60–3.48 (m, 2H, H-6a′,6b′), 1.85 (1s, 3H, OAc); ^{19}F NMR: δ –65.5. Anal. calcd. for $C_{39}H_{34}FN_5O_6$: C, 68.11, H, 4.98, F, 2.76, N, 10.18; found: C, 67.99, H, 4.74, F, 2.88, N, 10.08; ESI-MS (*m/z*): found 688.74 ($M + H^+$).

4.3.7. Synthesis of 9-(3-deoxy-3-fluoro-6-O-trityl-β-D-glycero-hex-2-enopyranosyl-4-ulose)-N^6-benzoyl adenine (8b)

Adenine derivative **8b** was synthesized from **7b** by the similar procedure as described for **8a**. Purified with flash chromatography using ethyl acetate–*n*-hexane (7:3) as eluent and it was obtained as a white foam. Yield: 0.8 g (50%), $R_f = 0.38$; ^{1}H NMR ($CDCl_3$): δ 9.05 (1H, br s, –NH), 8.88 and 8.27 (2H, 2s, H-2,8), 8.10–7.22 (m, 20H, Bz and 3C_6H_5), 7.07 (dtr, 1H, $J_{2',1'} = 1.4$ Hz, $J_{2',5'} = 1.9$ Hz, $J_{F,2'} = 6.7$ Hz, H-2′), 6.74 (dd, 1H, $J_{1',2'} = 1.4$ Hz, $J_{F,1'} = 10.2$ Hz, H-1′), 4.57 (m, 1H, H-5′), 3.73–3.66 (m, 2H, H-6a′,6b′); ^{19}F NMR: δ –64.3. Anal. calcd. for $C_{37}H_{28}FN_5O_4$: C, 71.03, H, 4.51, F, 3.04, N, 11.19; found: C, 71.19, H, 4.34, F, 3.25, N, 10.98. ESI-MS (*m/z*): found 626.66 ($M + H^+$).

4.4. Methods for measurement of biological activity

4.4.1. 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 units/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.

4.4.2. Nucleoside solutions

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 was used as a standard compound in antiviral experiments and 5FU as a standard compound in antitumor experiments.

4.4.3. Virus propagation

Rotavirus RF strain was propagated on Caco-2 monolayers in the presence of trypsin (1 μg per mL of DMEM) as described previously [84]. 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% units per mL ($TCID_{50}$/mL) was used (100 μL per well).

4.4.4. Antiviral assay

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

a) 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.

b) The neutralization of the virus in solution before attachment: Three-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.

4.4.5. Growth inhibition assay

It was performed on Caco-2 cell line by modified method described previously [85]. 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 to those in control wells ($n = 24$). The minimal inhibitory concentration (IC_{50}) of the tested compounds was obtained from the concentration–effect curve.

4.4.6. Cytotoxicity assay

Caco-2, H4, MCF-7, and skin melanoma cells (6×10^6 cells per 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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