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Note

Efficient synthesis of exomethylene- and keto-exomethylene- β -glucopyranosyl nucleoside analogs as potential cytotoxic agentsNiki Tzioumaki^a, Evangelia Tsoukala^a, Stella Manta^a, Christos Kiritsis^a, Jan Balzarini^b, Dimitri Komiotis^{a,*}^a Department of Biochemistry and Biotechnology, Laboratory of Organic Chemistry, University of Thessaly, 26 Ploutonos Str., 41221 Larissa, Greece^b Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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ABSTRACT

A novel series of exomethylene- and keto-exomethylene- β -glucopyranonucleosides with thymine, uracil, and 5-fluorouracil as heterocyclic bases have been designed and synthesized. Wittig condensation of the 3-keto glucoside **1** gave the corresponding 1,2:5,6-di-*O*-isopropylidene-3-deoxy-3-methylene- β -glucopyranose (**2**), which after hydrolysis and acetylation led to the precursor 1,2,4,6-tetra-*O*-acetyl-3-deoxy-3-methylene- β -glucopyranose (**4**). Compound **4** was condensed with silylated thymine, uracil, and 5-fluorouracil, respectively, deacetylated and acetalated to afford 1-(3'-deoxy-4',6'-*O*-isopropylidene-3'-methylene- β -*D*-glucopyranosyl)pyrimidines **7a–c**. Oxidation of the free hydroxyl group in the 2'-position of the sugar moiety led to the formation of the labile 1-(3'-deoxy-4',6'-*O*-isopropylidene-3'-methylene- β -*D*-glucopyranosyl-2'-ulose)pyrimidines **8a–c**. Finally, deisopropylidenation of the resulted derivatives **8a–c** afforded the diol nucleosides **9a–c**. The target keto-exomethylene analogs **9a–c** were more cytostatic against a variety of tumor cell lines than the corresponding saturated-hydroxy-exomethylene derivatives **6**. In particular, the 5-fluorouracil derivative **9c** was highly cytostatic at an IC₅₀ (50% inhibitory concentration) ranging between 0.56 and 9.4 μ g/mL, which was comparable to the free parental 5-fluorouracil base.

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The number of modified nucleosides has grown almost exponentially in the last decades, at least in part in response to the pressing need for new antitumor and antiviral agents.^{1–4} While several modified nucleosides have clinically verified utility in the treatment of cancer⁵ and virus infections, such as HIV,⁶ both the demonstration of toxic side effects and the emergence of resistant viral strains stimulate further studies directed toward the synthesis and biology of modified nucleosides.

Lately, nucleosides containing pyranosyl rings have been synthesized along with their analogs in order to develop antitumor,^{7,8} antiviral,^{9–13} antioxidant,¹⁴ and antimicrobial reagents.¹⁵ Experimental data also revealed that human poly(A)-specific ribonuclease¹⁶ and glycogen phosphorylase¹⁷ are among the molecular targets of these compounds. Modifications in the sugar residue of the pyranonucleosides furnished enzymatically stable nucleic acid analogs,^{18,19} which can be considered as bioisosters of natural nucleosides and nucleotides,²⁰ and can play a major role in different domains like therapy, diagnosis, and biotechnology.

In our continuing efforts to investigate new antiviral and antitumor agents, we have previously synthesized various unsaturated keto^{21–24} or exomethylene nucleosides.^{25–27} These uncommon pyranonucleosides proved to have a promising potential in combating rotaviral infections and exhibited cytostatic activity against various

cancer cell lines. Experimental evidence also suggested that these agents may act as novel types of 5-fluorouracil releasing prodrugs²⁸ that target thymidylate synthase for their cytostatic action.

With the above applications in mind and as continuation of our work in the synthesis of biologically active nucleosides, the aim of the present study is the stereocontrolled synthesis of pyranonucleosides, where we combined both the pharmacologically active keto group and exomethylene moiety in the sugar part to obtain new informations about structure–activity relationships. Therefore, we report herein the synthesis of a novel class of enone glucopyranonucleosides possessing an exomethylene group in the 3'-position and a keto group in the 2'-position of the sugar portion, containing thymine, uracil, and 5-fluorouracil as base moieties.

Retrosynthetic analysis suggested that the 3'-methylene nucleosides could be obtained from the corresponding 3'-keto nucleosides after Wittig olefination. However, the instability of the 3'-keto nucleosides, due to their susceptibility to β -elimination of the nucleobase^{29,30} prompted us to choose the appropriate 3-keto glucoside as the suitable precursor.

3'-Methylene-2'-keto- β -*D*-glucopyranonucleosides **9a–c** were prepared according to the synthetic route outlined in Scheme 1. Wittig condensation of the 1,2:5,6-di-*O*-isopropylidene- α -*D*-ribo-hexofuranos-3-ulose (**1**)³¹ with NaH and methyl triphenylphosphonium bromide (Ph₃P⁺CH₃Br[−]) at 0 °C, in the presence of *t*-amyl alcohol³² in tetrahydrofuran (THF), led to the formation of the desired 1,2:5,6-di-*O*-isopropylidene-3-deoxy-3-methylene- β -glucopyranose (**2**),³³ in

* Corresponding author. Tel.: +30 2410 565285; fax: +30 2410 565290.

E-mail address: dkom@bio.uth.gr (D. Komiotis).

Scheme 1. Reagents and conditions: (i) $Ph_3P^+CH_3Br^-$, NaH, *t*-amyl alcohol in THF, 0–25 °C; (ii) MeOH, H_2O , Amberlite IR-120 (H^+) resin; (iii) Ac_2O , pyridine; (iv) silylated base, $Me_3SiOSO_2CF_3/SnCl_4$, dry CH_3CN ; (v) ammonia/MeOH; (vi) acetone, 2,2-dimethoxypropane, *p*-toluenesulfonic acid; (vii) dry EtOAc/DMSO (4:1), Ac_2O , 98 °C, 8 min; (viii) dry CH_2Cl_2 , methanolic hydrogen chloride.

75% yield. Hydrolysis of **2** using Amberlite IR 120 (H^+) resin in methanol followed by acetylation using acetic anhydride (Ac_2O) in pyridine³¹ led to the 1,2,4,6-tetra-*O*-acetyl-3-deoxy-3-methylene- β -glucopyranose (**4**).

The protected, 1-(2',4',6'-tri-*O*-acetyl-3'-deoxy-3'-methylene- β -D-glucopyranosyl) nucleosides of thymine (**5a**), uracil (**5b**) and 5-fluorouracil (**5c**), were obtained upon condensation of the precursor material **4** with silylated thymine, uracil and 5-fluorouracil, respectively, in the presence of tin chloride(IV) and trimethylsilyl trifluoromethane-sulfonate ($Me_3SiOSO_2CF_3$) in refluxing acetonitrile.³⁴ Removal of all *O*-acetyl protecting groups of **5a–c**, with saturated methanolic ammonia,³⁵ gave **6a–c** in excellent yields. Specific acetalation of the aforementioned compounds using 2,2-dimethoxypropane³⁵ in acetone led to the 2',3'-*O*-isopropylidene derivatives **7a–c**, in yields 72%, 75%, and 68%, respectively. The 1H NMR spectra of **7a–c** showed the presence of two vinylic protons at 5.40–5.34 ppm and 5.35–5.32 ppm, each as a broad singlet, corresponding to the 3'-exomethylene groups.

The crucial step of the synthetic pathway proved to be the oxidation of the suitably protected precursors **7a–c**. Therefore, oxidation of **7a–c** with the Pfaltzner and Moffatt³⁶ [dimethyl sulfoxide (DMSO)/*N,N'*-dicyclohexylcarbodiimide (DCC)],^{37,38} Garegg and Samuelsson³⁹ (CrO_3 /pyridine/ Ac_2O),⁴⁰ Swern⁴¹ (DMSO/oxalyl chloride),^{42,43} and pyridinium dichromate (PDC)/3E molecular sieves⁴⁴ reagents resulted in β -elimination of the nucleobase, while a mixture of untreatable products was obtained. Dess–Martin⁴⁵ (12-*I*-5-periodinane) oxidation³⁰ of **7a–c** afforded the desired methylene ketonucleosides **8a–c** but unfortunately they could not be separated from the iodo-compound byproduct.

However, oxidation performed by a modified Albright–Goldman method using the DMSO/EtOAc/ Ac_2O system⁴⁶ led to the

target 1-(3'-deoxy-4',6'-*O*-isopropylidene-3'-methylene- β -D-glucopyranosyl-2'-ulose)nucleosides **8a–c**, well characterized by 1H NMR and mass spectroscopy. Even though the desired compounds were readily available after isolation, they seemed to be labile during storage. Therefore, they were immediately treated with methanolic hydrogen chloride⁴⁷ to give the diol nucleosides **9a–c**, in yields 63%, 60%, and 55%, respectively. It should be mentioned that treatment of **8a–c** with a mixture of $CH_2Cl_2/HCOOH$, 1:1, or 90% trifluoroacetic acid in methanol (MeOH), or pyridinium *p*-toluenesulfonate (PPTS) in MeOH,⁴⁸ or Amberlite IR 120 (H^+) resin,⁴⁹ or *p*-toluenesulfonic acid in MeOH⁵⁰ unfortunately did not afford the target nucleosides **9a–c** but it led instead to intractable materials.

The target nucleosides **9a–c** and their key intermediates saturated exomethylene **6a–c** were tested for their inhibitory effects on the proliferation of murine leukemia cells (L1210), human T-lymphocyte cells (CEM), and human cervix carcinoma cells (HeLa).

In contrast to the previously synthesized saturated 2'- or 4'-exo-methylene nucleosides,^{25–27} no marked cytostatic activity was noticed for the newly 3'-exomethylene analogs **6a–c** (Table 1). It appears that the transposition of the methylene moiety from C-2' or C-4' to C-3' lowers the cytotoxic activity of this type of molecules. On the other hand, the target keto-exomethylene derivatives **9a–c** were endowed with an antiproliferative activity, in particular the 5-fluorouracil derivative **9c**, indicating the important role of the keto group in the eventual cytostatic activity of these compounds. Compound **9c** showed a very similar cytostatic activity as the free base 5-fluorouracil (5-FU), and therefore, we assume that the compound acts as an efficient prodrug of 5-FU. Our results enforce the importance of the presence of the keto-group together

Table 1

Inhibitory effects on the proliferation of murine leukemia cells (L1210), human T-lymphocyte cells (CEM), and human cervix carcinoma cells (HeLa)

Compound	IC ₅₀ ^a (μg/ml)		
	L1210	CEM	HeLa
6a	>200	>200	>200
9a	77 ± 24	126 ± 34	73
6b	>200	>200	>200
9b	84 ± 10	101 ± 14	92
6c	>200	>200	>200
9c	0.56 ± 0.21	9.4 ± 3.0	1.5 ± 0.0
5-Fluorouracil	0.49 ± 0.13	18 ± 5	0.54 ± 0.12

^a 50% Inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.

with the carbon–carbon double bond in the sugar moiety of the nucleoside analogs. This conjugation proved to be once again a prerequisite for biological activity.^{21–28} It appears that the unsaturated keto system might function as an acceptor in a Michael-addition type mechanism.²⁷

1. Experimental

1.1. General procedure

Melting points were recorded in a Mel-Temp apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on Merck precoated 60F₂₅₄ 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). ¹H and ¹³C NMR spectra were obtained at room temperature with a Bruker 400 spectrometer at 400 and 100 MHz, respectively, using chloroform-*d* (CDCl₃), methanol-*d*₄ (CD₃OD), and acetone-*d*₆ ((CD₃)₂CO). 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 (ESIMS). Optical rotations were measured using Autopol I polarimeter.

All reactions were carried out in dry solvents. CH₂Cl₂ was distilled from phosphorous pentoxide and stored over 4E molecular sieves. Acetonitrile and toluene (Tol) 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.

1.2. Synthesis of 1,2,4,6-tetra-*O*-acetyl-3-deoxy-3-methylene-*D*-glucopyranose (**4**)

1.2.1. 1,2:5,6-Di-*O*-isopropylidene-3-deoxy-3-methylene-*D*-glucofuranose (**2**)

To a stirred suspension of Ph₃P⁺CH₃Br[−] (54.48 mmol, 19.50 g) and *t*-amyl alcohol (59.44 mmol, 6.51 mL) in dry THF (268.11 mL) was added NaH (95.10 mmol, 2.28 g, 60% in oil) at 0 °C and the reaction mixture was stirred for 2 h at ambient temperature under nitrogen. To this yellow phosphorous ylide was added a solution of 1,2:5,6-di-*O*-isopropylidene- α -*D*-ribo-hexofuranos-3-ulose (**1**)³¹ (16.51 mmol, 4.26 g) in dry THF (26.28 mL) dropwise, at 0 °C under nitrogen. After the mixture was stirred for 30 min at ambient temperature, 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 (hexane, *R*_f 0.2) to give 3.17 g (75%) of compound **2** as a white solid: mp 85–87 °C (hexane–EtOAc); [α]_D²² +2 (c 0.3, CHCl₃); The ¹H NMR data agreed with the assignments⁵¹ corrected from the initial report;⁵² Anal. Calcd for C₁₃H₂₀O₅: C, 60.92; H, 7.87. Found: C, 60.98; H, 7.83. ESIMS: *m/z* 257.3 [M+H⁺].

1.2.2. 3-Deoxy-3-methylene-*D*-glucopyranose (**3**)

To a solution of **2** (12.38 mmol, 3.17 g) in MeOH (19.5 mL) and H₂O (110.6 mL) was added Amberlite IR 120 (H⁺) resin and the mixture was refluxed overnight. The reaction mixture was filtered and evaporated to dryness to give 1.85 g (85%) of compound **3** as a viscous oil and it was used without further purification (1:9 MeOH–EtOAc, *R*_f 0.4); [α]_D²² −8 (c 0.5, MeOH); Anal. Calcd for C₇H₁₂O₅: C, 47.72; H, 6.87. Found: C, 47.86; H, 6.82. ESIMS: *m/z* 177.2 [M+H⁺].

1.2.3. 1,2,4,6-Tetra-*O*-acetyl-3-deoxy-3-methylene-*D*-glucopyranose (**4**)

Compound **3** (10.53 mmol, 1.85 g) was dissolved in a mixture of pyridine (37.1 mL) and Ac₂O (19.4 mL). The reaction was carried out at room temperature for 1 h, then was quenched with MeOH at 0 °C, and was concentrated in vacuum. The residue was diluted with EtOAc and washed with saturated sodium bisulfate, sodium bicarbonate, and water. The organic extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness to give 3.55 g (98%) of tetraacetylated **4**, (0.5:9.5 MeOH–EtOAc, *R*_f 0.54) as a white foam: [α]_D²² +10 (c 0.5, CHCl₃); Anal. Calcd for C₁₅H₂₀O₉: C, 52.32; H, 5.85. Found: C, 52.36; H, 5.92. ESIMS: *m/z* 345.3 [M+H⁺].

1.3. Synthesis of 1-(3'-deoxy-3'-methylene- β -*D*-glucopyranosyl-2'-ulose)thymine (**9a**)

1.3.1. 1-(2',4',6'-Tri-*O*-acetyl-3'-deoxy-3'-methylene- β -*D*-glucopyranosyl)thymine (**5a**)

A mixture of thymine (14.45 mmol, 1.82 g), hexamethyldisilazane (HMDS) (17.92 mmol, 3.78 mL), and saccharin (0.66 mmol, 121.76 mg) in dry CH₃CN (47 mL) was refluxed for 30 min under nitrogen. To this were added 1,2,4,6-tetra-*O*-acetyl-3-deoxy-3-methylene-*D*-glucopyranose (**4**) (10.32 mmol, 3.55 g) and SnCl₄ (14.45 mmol, 1.7 mL). The reaction mixture was refluxed for 1 h, neutralized with saturated sodium bicarbonate, and then extracted with CH₂Cl₂. The organic extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was purified by flash chromatography (4:6 hexane–EtOAc, *R*_f 0.4) to give 2.33 g (55%) of compound **5a** as a white solid: mp 102–104 °C (hexane–EtOAc); [α]_D²² −2 (c 0.5, CHCl₃); λ _{max} (CHCl₃) 263 nm (ϵ 8502); ¹H NMR (CDCl₃, 400 MHz): δ 8.17 (br s, 1H, NH), 7.19 (s, 1H, H-6), 5.67 (d, 1H, *J*_{1',2'} 9.3 Hz, H-1'), 5.50 (d, 1H, H-2'), 5.37 (d, 1H, *J*_{4',5'} 9.6 Hz, H-4'), 5.21, 5.12 (2s, 2H, methylene), 4.34–4.15 (m, 2H, H-6a', H-6b'), 3.80–3.75 (m, 1H, H-5'), 2.19, 2.11, 2.10 (3s, 9H, 3OAc), 1.96 (s, 3H, 5-CH₃). Anal. Calcd for C₁₈H₂₂N₂O₉: C, 52.68; H, 5.40; N, 6.83. Found: C, 52.73; H, 5.34; N, 6.82. ESIMS *m/z* 411.4 [M+H⁺].

1.3.2. 1-(3'-Deoxy-3'-methylene- β -*D*-glucopyranosyl)thymine (**6a**)

Compound **5a** (5.67 mmol, 2.33 g) was treated with ammonia/MeOH (saturated at 0 °C, 240 mL). The solution was stirred overnight at room temperature and then was concentrated under reduced pressure. The residue was purified by flash chromatography (2:8 MeOH–EtOAc, *R*_f 0.45) to give 1.55 g (96%) of compound **6a**, as a white foam: [α]_D²² −2 (c 0.3, MeOH); λ _{max} (MeOH) 263 nm (ϵ 6322); ¹H NMR (CD₃OD, 400 MHz): δ 7.65 (s, 1H, H-6), 5.39 (s, 2H, methylene), 5.36 (d, 1H, *J*_{1',2'} 9.2 Hz, H-1'), 4.26 (d, 1H, H-2'), 4.12 (d, 1H, *J*_{4',5'} 9.6 Hz, H-4'), 3.92–3.79 (m, 2H, H-6a', H-6b'), 3.43–3.29 (m, 1H, H-5'), 1.93 (s, 3H, 5-CH₃); ¹³C NMR (CD₃OD, 100 MHz): 152.0, 150.5, 149.0, 137.2, 110.5, 104.8, 85.1, 82.5, 69.5, 67.2, 61.3, 10.9. Anal. Calcd for C₁₂H₁₆N₂O₆: C, 50.70; H, 5.67; N, 9.85. Found: C, 50.91; H, 5.59; N, 9.73. ESIMS *m/z* 285.3 [M+H⁺].

1.3.3. 1-(3'-Deoxy-4',6'-O-isopropylidene-3'-methylene-β-D-glucopyranosyl)thymine (7a)

To a stirred suspension of **6a** (5.45 mmol, 1.55 g) in anhydrous acetone (93.12 mL) and 2,2-dimethoxypropane (4.87 mL) was added *p*-toluenesulfonic acid monohydrate (0.93 mmol, 0.176 g). After 3 h the resulting solution was neutralized with triethylamine so that pH did not exceed 7. The solution was concentrated and the residue was purified by flash chromatography (4:6 hexane–EtOAc, R_f 0.26) to give 1.27 g (72%) of **7a** as a white solid: mp 167–169 °C (hexane–EtOAc); $[\alpha]_D^{22} +8$ (c 0.5, CHCl₃); $\lambda_{\max}$ (CHCl₃) 265 nm (ϵ 9477); ¹H NMR (CDCl₃, 400 MHz): δ 8.31 (br s, 1H, NH), 7.16 (s, 1H, H-6), 5.51 (d, 1H, $J_{1,2'}$ 8.9 Hz, H-1'), 5.40, 5.33 (2s, 2H, methylene), 4.10–4.04 (m, 2H, H-2', H-4'), 3.97–3.76 (m, 2H, H-6a', H-6b'), 3.44–3.36 (m, 1H, H-5'), 1.91 (s, 3H, 5-CH₃), 1.56, 1.50 (2s, 6H, 2CH₃). Anal. Calcd for C₁₅H₂₀N₂O₆: C, 55.55; H, 6.22; N, 8.64. Found: C, 55.61; H, 6.34; N, 8.58. ESIMS m/z 325.3 [M+H⁺].

1.3.4. 1-(3'-Deoxy-4',6'-O-isopropylidene-3'-methylene-β-D-glucopyranosyl-2'-ulose)thymine (8a)

A mixture of **7a** (3.92 mmol, 1.27 g) and Ac₂O (101.53 mmol, 9.58 mL) was stirred in 15 mL of dry DMSO/EtOAc (4/1) for 8 min under nitrogen at 98 °C and then was concentrated in vacuum. The reaction mixture was extracted with EtOAc, the organic extract was washed with water dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was purified by flash chromatography (1:1 hexane–EtOAc, R_f 0.3) to give 0.61 g (48%) of compound **8a** as a white foam, which was found to undergo decomposition during storage: ¹H NMR (CDCl₃, 400 MHz): δ 8.53 (br s, 1H, NH), 6.97 (s, 1H, H-6), 6.32, 5.83 (2s, 2H, methylene), 5.37 (s, 1H, H-1'), 4.69 (d, 1H, $J_{4,5'}$ 10.0 Hz, H-4'), 4.00–3.64 (m, 3H, H-5', H-6a', H-6b'), 1.93 (s, 3H, 5-CH₃), 1.58, 1.49 (2s, 6H, 2CH₃). Anal. Calcd for C₁₅H₁₈N₂O₆: C, 55.90; H, 5.63; N, 8.69. Found: C, 55.69; H, 5.37; N, 8.58. ESIMS m/z 323.3 [M+H⁺].

1.3.5. 1-(3'-Deoxy-3'-methylene-β-D-glucopyranosyl-2'-ulose)thymine (9a)

To a solution of **8a** (1.88 mmol, 0.61 g) in dry CH₂Cl₂ (3.7 mL) was added methanolic hydrogen chloride (3.7 mL), which was obtained from acetyl chloride (0.37 mL) in dry MeOH (9.4 mL) at 0 °C. After the mixture was stirred overnight, the solution was concentrated, and the residue was purified by flash chromatography (EtOAc, R_f 0.35) to give 0.34 g (63%) of **9a** as a white foam: $[\alpha]_D^{22} -26$ (c 0.5, CHCl₃); $\lambda_{\max}$ (CHCl₃) 260 nm (ϵ 7004); ¹H NMR ((CD₃)₂CO, 400 MHz): δ 8.51 (br s, 1H, NH), 7.35 (s, 1H, H-6), 7.21 (br s, 1H, H-1'), 6.22 (br s, 1H, H-4'), 4.93 (br s, 1H, H-5'), 4.11 (s, 2H, methylene), 3.86–3.76 (m, 2H, H-6a', H-6b'), 1.92 (s, 3H, 5-CH₃); ¹³C NMR ((CD₃)₂CO, 100 MHz): 189.1, 145.3, 141.6, 135.6, 102.3, 101.5, 82.5, 76.2, 67.6, 63.7, 57.9, 10.9. Anal. Calcd for C₁₂H₁₄N₂O₆: C, 51.06; H, 5.00; N, 9.93. Found: C, 51.34; H, 4.92; N, 9.99. ESIMS m/z 283.3 [M+H⁺].

1.4. Synthesis of 1-(3'-deoxy-3'-methylene-β-D-glucopyranosyl-2'-ulose)uracil (9b)

1.4.1. 1-(2',4',6'-Tri-O-acetyl-3'-deoxy-3'-methylene-β-D-glucopyranosyl)uracil (5b)

A mixture of uracil (14.00 mmol, 1.57 g), HMDS (17.36 mmol, 3.67 mL), and saccharin (0.64 mmol, 117.97 mg) in dry CH₃CN (44 mL) was refluxed for 30 min. To this were added 1,2,4,6-tetra-O-acetyl-3-deoxy-3-methylene-β-D-glucopyranose (**4**), (10.00 mmol, 3.44 g), and SnCl₄ (14.00 mmol, 1.6 mL). The reaction mixture was refluxed at 80 °C for 1 h, neutralized with saturated sodium bicarbonate, and then extracted with CH₂Cl₂. The organic extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was purified by flash chromatography

(4:6 hexane–EtOAc, R_f 0.3) to give 2.1 g (53%) of compound **5b**, as a white foam: $[\alpha]_D^{22} -2$ (c 0.5, CHCl₃); $\lambda_{\max}$ (CHCl₃) 257 nm (ϵ 6139); ¹H NMR (CDCl₃, 400 MHz): δ 8.08 (br s, 1H, NH), 7.38 (d, 1H, $J_{5,6}$ 8.2 Hz, H-6), 5.82 (d, 1H, H-5), 5.67 (d, 1H, $J_{1,2'}$ 9.2 Hz, H-1'), 5.48 (d, 1H, H-2'), 5.36 (d, 1H, $J_{4,5'}$ 9.8 Hz, H-4'), 5.23, 5.12 (2s, 2H, methylene), 4.34–4.15 (m, 2H, H-6a', H-6b'), 3.79–3.77 (m, 1H, H-5'), 2.19, 2.12, 2.10 (3s, 9H, 3OAc). Anal. Calcd for C₁₇H₂₀N₂O₉: C, 51.52; H, 5.09; N, 7.07. Found: C, 51.59; H, 5.27; N, 7.01. ESIMS m/z 397.4 [M+H⁺].

1.4.2. 1-(3'-Deoxy-3'-methylene-β-D-glucopyranosyl)uracil (6b)

Uracil derivative **6b** was synthesized from **5b** by the similar procedure as described for **6a**. It was purified by flash chromatography (EtOAc, R_f 0.25) to give 1.3 g (90%) of compound **6b**, as a white foam: $[\alpha]_D^{22} +2$ (c 0.1, MeOH); $\lambda_{\max}$ (MeOH) 261 nm (ϵ 7795); ¹H NMR (CD₃OD, 400 MHz): δ 7.81 (d, 1H, $J_{5,6}$ 8.1 Hz, H-6), 5.76 (d, 1H, H-5), 5.39 (s, 2H, methylene), 5.36 (d, 1H, $J_{1,2'}$ 9.2 Hz, H-1'), 4.23 (d, 1H, H-2'), 4.10 (d, 1H, $J_{4,5'}$ 9.6 Hz, H-4'), 3.92–3.78 (m, 2H, H-6a', H-6b'), 3.33–3.31 (m, 1H, H-5'); ¹³C NMR (CD₃OD, 100 MHz): 153.1, 151.2, 150.0, 142.7, 106.3, 103.1, 86.6, 84.1, 71.2, 68.6, 62.7. Anal. Calcd for C₁₁H₁₄N₂O₆: C, 48.89; H, 5.22; N, 10.37. Found: C, 48.85; H, 5.29; N, 10.43. ESIMS m/z 271.3 [M+H⁺].

1.4.3. 1-(3'-Deoxy-4',6'-O-isopropylidene-3'-methylene-β-D-glucopyranosyl)uracil (7b)

Uracil derivative **7b** was synthesized from **6b** by the similar procedure as described for **7a**. It was purified by flash chromatography (EtOAc, R_f 0.15) to give 1.11 g (75%) of compound **7b** as a white solid: mp 179–181 °C (hexane–EtOAc); $[\alpha]_D^{22} +4$ (c 0.5, CHCl₃); $\lambda_{\max}$ (CHCl₃) 261 nm (ϵ 7314); ¹H NMR (CDCl₃, 400 MHz): δ 8.46 (br s, 1H, NH), 7.37 (d, 1H, $J_{5,6}$ 8.1 Hz, H-6), 5.76 (d, 1H, H-5), 5.46 (d, 1H, $J_{1,2'}$ 8.9 Hz, H-1'), 5.34, 5.32 (2s, 2H, methylene), 4.14 (d, 1H, H-2'), 4.04 (d, 1H, $J_{4,5'}$ 9.5 Hz, H-4'), 3.96–3.76 (m, 2H, H-6a', H-6b'), 3.39–3.34 (m, 1H, H-5'), 1.55, 1.50 (2s, 6H, 2CH₃). Anal. Calcd for C₁₄H₁₈N₂O₆: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.35; H, 5.89; N, 9.39. ESIMS m/z 311.30 [M+H⁺].

1.4.4. 1-(3'-Deoxy-4',6'-O-isopropylidene-3'-methylene-β-D-glucopyranosyl-2'-ulose)uracil (8b)

Uracil derivative **8b** was synthesized from **7b** by the similar procedure as described for **8a**. It was purified by flash chromatography (1:1 hexane–EtOAc, R_f 0.2) to give 0.61 g (55%) of compound **8b** as a white foam, which was found to undergo decomposition during storage: ¹H NMR (CDCl₃, 400 MHz): δ 8.33 (br s, 1H, NH), 7.15 (d, 1H, $J_{5,6}$ 7.9 Hz, H-6), 6.32, 5.84 (2s, 2H, methylene), 5.76 (d, 1H, H-5), 5.36 (s, 1H, H-1'), 4.69 (d, 1H, $J_{4,5'}$ 10.0 Hz, H-4'), 4.30–3.64 (m, 3H, H-5', H-6a', H-6b'), 1.59, 1.49 (2s, 6H, 2CH₃). Anal. Calcd for C₁₄H₁₆N₂O₆: C, 54.54; H, 5.23; N, 9.09. Found: C, 54.63; H, 5.30; N, 9.17. ESIMS m/z 309.3 [M+H⁺].

1.4.5. 1-(3'-Deoxy-3'-methylene-β-D-glucopyranosyl-2'-ulose)uracil (9b)

Uracil derivative **9b** was synthesized from **8b** by the similar procedure as described for **9a**. It was purified by flash chromatography (EtOAc, R_f 0.1) to give 0.32 g (60%) of **9b** as a white foam: $[\alpha]_D^{22} +2$ (c 0.1, MeOH); $\lambda_{\max}$ (MeOH) 259 nm (ϵ 7220); ¹H NMR ((CD₃)₂CO, 400 MHz): δ 8.51 (br s, 1H, NH), 7.46 (d, 1H, $J_{5,6}$ 8.0 Hz, H-6), 7.21 (br s, 1H, H-1'), 6.29 (br s, 1H, H-4'), 5.65 (d, 1H, H-5), 4.97–4.91 (m, 1H, H-5'), 4.12 (br s, 2H, methylene), 3.85–3.81 (m, 2H, H-6a', H-6b'); ¹³C NMR ((CD₃)₂CO, 100 MHz): 189.4, 144.8, 142.1, 135.1, 102.2, 100.5, 82.4, 76.4, 67.4, 63.6, 57.8. Anal. Calcd for C₁₁H₁₂N₂O₆: C, 49.26; H, 4.51; N, 10.44. Found: C, 49.33; H, 4.82; N, 10.68. ESIMS m/z 269.2 [M+H⁺].

1.5. Synthesis of 1-(3'-deoxy-3'-methylene-β-D-glucopyranosyl-2'-ulose)5-fluorouracil (9c)

1.5.1. 1-(2',4',6'-Tri-O-acetyl-3'-deoxy-3'-methylene-β-D-glucopyranosyl)5-fluorouracil (5c)

A mixture of 5-fluorouracil (13.92 mmol, 1.81 g), HMDS (17.26 mmol, 3.64 mL), and saccharin (0.64 mmol, 117.29 mg) in dry CH₃CN (68 mL) was refluxed for 30 min. To this was added 1,2,4,6-tetra-O-acetyl-3-deoxy-3-methylene-β-D-glucopyranose (**4**), (10.71 mmol, 3.7 g), and Me₃SiOSO₂CF₃ (14.99 mmol, 2.7 mL). The reaction mixture was refluxed at 65 °C for 1.5 h, neutralized with saturated sodium bicarbonate, and then extracted with CH₂Cl₂. The organic extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was purified by flash chromatography (4:6 hexane–EtOAc, *R_f* 0.2) to give 2.57 g (58%) of compound **5c** as a white foam: $[\alpha]_D^{22} +2$ (c 0.3, CHCl₃); $\lambda_{\max}$ (CHCl₃) 261 nm (ϵ 7446); ¹H NMR (CDCl₃, 400 MHz): δ 8.43 (br s, 1H, NH), 7.46 (d, 1H, *J*_{F5,6} 5.5 Hz, H-6), 5.64 (d, 1H, *J*_{1',2'} 9.0 Hz, H-1'), 5.44 (d, 1H, H-2'), 5.36 (d, 1H, *J*_{4',5'} 9.8 Hz, H-4'), 5.25, 5.14 (2s, 2H, methylene), 4.34–4.15 (m, 2H, H-6a', H-6b'), 3.80–3.78 (m, 1H, H-5'), 2.19, 2.13, 2.11 (3s, 9H, 3OAc). Anal. Calcd for C₁₇H₁₉FN₂O₉: C, 49.28; H, 4.62; N, 6.76. Found: C, 49.41; H, 4.32; N, 6.83. ESIMS *m/z* 415.3 [M+H⁺].

1.5.2. 1-(3'-Deoxy-3'-methylene-β-D-glucopyranosyl)5-fluorouracil (6c)

5-Fluorouracil analog **6c** was synthesized from **5c** by the similar procedure as described for **6a**. The residue was chromatographed (EtOAc, *R_f* 0.22) and 1.65 g (92%) of **6c** was obtained as a white foam: $[\alpha]_D^{22} +10$ (c 0.7, MeOH); $\lambda_{\max}$ (MeOH) 269 nm (ϵ 6019); ¹H NMR (CD₃OD, 400 MHz): δ 8.02 (d, 1H, *J*_{F5,6} 6.7 Hz, H-6), 5.39 (s, 2H, methylene), 5.35 (d, 1H, *J*_{1',2'} 9.1 Hz, H-1'), 4.20 (d, 1H, H-2'), 4.11 (d, 1H, *J*_{4',5'} 11.5 Hz, H-4'), 3.92–3.77 (m, 2H, H-6a', H-6b'), 3.31–3.28 (m, 1H, H-5'); ¹³C NMR (CD₃OD, 100 MHz): 150.2, 149.0, 132.3, 131.7, 128.6, 105.2, 85.7, 82.5, 69.6, 67.2, 61.2. Anal. Calcd for C₁₁H₁₃FN₂O₆: C, 45.84; H, 4.55; N, 9.72. Found: C, 45.11; H, 4.27; N, 9.38. ESIMS *m/z* 289.3 [M+H⁺].

1.5.3. 1-(3'-Deoxy-4',6'-O-isopropylidene-3'-methylene-β-D-glucopyranosyl)5-fluorouracil (7c)

5-Fluorouracil analog **7c** was synthesized from **6c** by the similar procedure as described for **7a**. It was purified by flash chromatography (6:4 hexane–EtOAc, *R_f* 0.2) to give 1.27 g (68%) of compound **7c** as a white foam: $[\alpha]_D^{22} +8$ (c 0.4, CHCl₃); UV $\lambda_{\max}$ (CHCl₃) 267 nm (ϵ 6018); ¹H NMR (CDCl₃, 400 MHz): δ 9.64 (br s, 1H, NH), 7.44 (d, 1H, *J*_{F5,6} 5.3 Hz, H-6), 5.48 (d, 1H, *J*_{1',2'} 8.8 Hz, H-1'), 5.37, 5.35 (2s, 2H, methylene), 4.11 (d, 1H, H-2'), 4.04 (d, 1H, *J*_{4',5'} 9.4 Hz, H-4'), 3.96–3.77 (m, 2H, H-6a', H-6b'), 3.42–3.37 (m, 1H, H-5'), 1.55, 1.49 (2s, 6H, 2CH₃). Anal. Calcd for C₁₄H₁₇FN₂O₆: C, 51.22; H, 5.22; N, 8.53. Found: C, 51.29; H, 5.41; N, 8.47. ESIMS *m/z* 329.3 [M+H⁺].

1.5.4. 1-(3'-Deoxy-4',6'-O-isopropylidene-3'-methylene-β-D-glucopyranosyl-2'-ulose)5-fluorouracil (8c)

5-Fluorouracil analog **8c** was synthesized from **7c** by the similar procedure as described for **8a**. Chromatography of the residue (1:1 hexane–EtOAc, *R_f* 0.45) gave 0.58 g (46%) of compound **8c** as a white foam, which was found to undergo decomposition during storage: ¹H NMR (CDCl₃, 400 MHz): δ 9.18 (br s, 1H, NH), 7.37 (d, 1H, *J*_{F5,6} 5.3 Hz, H-6), 6.32, 5.83 (2s, 2H, methylene), 5.32 (m, 1H, H-1'), 4.67 (d, 1H, *J*_{4',5'} 10.0 Hz, H-4'), 4.24–3.66 (m, 3H, H-5', H-6a', H-6b'), 1.59, 1.51 (2s, 6H, 2CH₃). Anal. Calcd for C₁₄H₁₅FN₂O₆: C, 51.54; H, 4.63; N, 8.59. Found: C, 51.69; H, 4.74; N, 8.89. ESIMS *m/z* 327.3 [M+H⁺].

1.5.5. 1-(3'-Deoxy-3'-methylene-β-D-glucopyranosyl-2'-ulose)5-fluorouracil (9c)

5-Fluorouracil derivative **9c** was synthesized from **8c** by the similar procedure as described for **9a**. It was purified by flash chromatography (0.5: 9.5 MeOH–CH₂Cl₂, *R_f* 0.35) to give 0.28 g (55%) of compound **9c** as a white foam: $[\alpha]_D^{22} -26$ (c 0.4, MeOH); $\lambda_{\max}$ (MeOH) 262 nm (ϵ 9525); ¹H NMR ((CD₃)₂CO, 400 MHz): δ 7.73 (d, 1H, *J*_{F5,6} 6.6 Hz, H-6), 7.21 (br s, 1H, H-1'), 6.31 (br s, 1H, H-4'), 4.98 (br s, 1H, H-5'), 4.12 (s, 2H, methylene), 3.88–3.84 (m, 2H, H-6a', H-6b'); ¹³C NMR ((CD₃)₂CO, 100 MHz): 189.2, 145.1, 134.6, 127.1, 108.1, 103.3, 82.1, 76.6, 67.9, 63.7, 58.0. Anal. Calcd for C₁₁H₁₁FN₂O₆: C, 46.16; H, 3.87; N, 9.79. Found: C, 46.25; H, 3.89; N, 9.86. ESIMS *m/z* 287.2 [M+H⁺].

1.6. Cytostatic activity assays

Murine leukemia L1210, human lymphocyte CEM, and human cervix carcinoma HeLa cells were seeded in 96-well microtiter plates at 50,000 (L1210), 75,000 (CEM) or 20,000 (HeLa) cells per 200 μL-well in the presence of different concentrations of the test compounds. After 2 (L1210), 3 (CEM) or 4 (HeLa) days, the viable cell number was counted using a Coulter counter apparatus. The 50% cytostatic concentration (CC₅₀) was defined as the compound concentration required to inhibit tumor cell proliferation by 50%.

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