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Synthesis and antiviral activities of new acyclic and "double-headed" nucleoside analogues

Xinying Zhang [a,1], Adel Amer [a,2], Xuesen Fan [a], Jan Balzarini [b], Johan Neyts [b], Erik De Clercq [b], Mark Prichard [c], Earl Kern [c], Paul F. Torrence [a,*]

[a] *Department of Chemistry and Biochemistry, Northern Arizona University, Flagstaff, AZ 86011-5698, USA*
[b] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, 3000 Leuven, Belgium*
[c] *Department of Pediatrics, University of Alabama School of Medicine, Birmingham, AL, USA*

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Abstract

To develop an understanding of the structure–activity relationships for the inhibition of orthopoxviruses by nucleoside analogues, a variety of novel chemical entities were synthesized. These included a series of pyrimidine 5-hypermodified acyclic nucleoside analogues based upon recently discovered new leads, and some previously unknown "double-headed" or "abbreviated" nucleosides. None of the synthetic products possessed significant activity against two representative orthopoxviruses; namely, vaccinia virus and cowpox virus. They were also devoid of significant activity against a battery of other DNA and RNA viruses. So far as the results with the orthopoxviruses and herpes viruses, the results may point to the necessity for nucleoside analogues 5′-phosphorylation for antiviral efficacy.

Keywords: Smallpox; Vaccinia virus; Cowpox virus; Thymidine kinase

* Corresponding author. Fax: +1 928 523 8111.
E-mail address: Paul.Torrence@nau.edu (P.F. Torrence).
[1] On leave from the School of Chemistry and Environmental Sciences, Henan Normal University, Xinxiang, Henan 453007, China.
[2] On leave from the Department of Chemistry, Faculty of Science, Alexandria University, Egypt.

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1. Introduction

The threat of bioterrorism has mandated that drug countermeasures be developed against the ancient scourge of smallpox caused by the variola orthopoxvirus [1–5]. A priority of the US Government is to have two food and drug administration (FDA) approved agents available with two more in the drug pipeline [6].

One drug, Cidofovir (Vistide®), licensed to treat cytomegalovirus (CMV) retinitis in HIV-infected patients, is available through a special protocol (Investigational New Drug, IND) for emergency treatment of smallpox or vaccine reactions (http://www.bt.cdc.gov/agent/smallpox/vaccination/cidofovir.asp) if vaccine immune globulin (VIG) is not effective [7–10]. Some entities for the treatment of orthopoxvirus infections in pre-clinical or clinical development include inhibitors of viral morphogenesis (TTP-6171) [11] and viral release (ST-246) [12] as well as compounds interacting with cellular targets [i.e. Erb-1 kinase inhibitors (CI-1033) [13,14] and tyrosine kinase inhibitors (Gleevec, STI-571)] [15].

Recently, we have described several novel 5-substituted pyrimidine nucleosides (Fig. 1, **2a**–**2c**) all derived from the 5-formyl-2′-deoxyuridine (Fig. 1, **1**) building block [16–19]. In an attempt to improve upon these leads, we have prepared a series of analogues based upon a structural motif that has been successful in other domains of antiviral chemotherapy. We chose the acyclic nucleoside analogue structural motif; specifically, the 2-hydroxyethoxymethyl substituent, exemplified by acyclovir [20,21]. Although the lead compounds of Fig. 1 have been found active [16–19] against only orthopoxviruses, vaccinia virus and cowpox virus, and some herpes viruses (Prichard et al., unpublished observations), to be judicious, the synthetic products of this study were evaluated against a wide range of RNA and DNA viruses.

In a second structural modification, we have synthesized several unusual "double-headed" nucleosides. There are relatively few publications on the synthesis of these so called

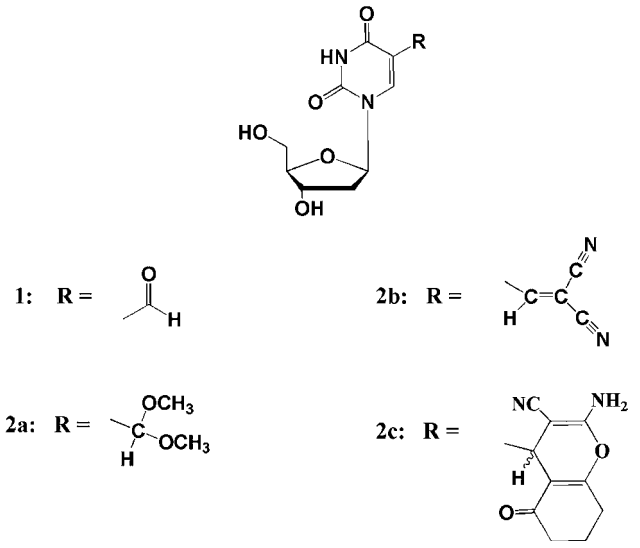


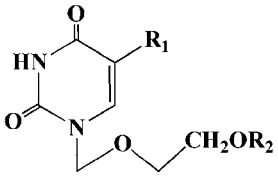
Fig. 1. Structures of key intermediate (**1**) and nucleosides (**2a**, **2b**, and **2c**) with established anti-orthopoxvirus activities.

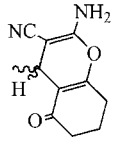
"double-headed nucleosides" or "abbreviated nucleosides". Nelson Leonard and associates [22,23], and in a separate simultaneous effort, Shen and colleagues [24] at Merck provided the first syntheses and characterizations of such molecules. Since then a number of publications, a few of which are referenced here [25–29], have followed Leonard's and Shen's leads.

While these "double-headed" nucleosides, in the embodiment presented in this study, likely would not be phosphorylated by cellular or viral kinases because of the missing 5′-hydroxy moiety, they might be expected to exhibit promising biological activities for other reasons. 1,2,4-Triazoles have demonstrated antiviral activity against HIV-1 [30,31]. In addition, the closely related 1,2,3-triazole and benzotriazoles have shown activity as xanthine oxidase inhibitors, antimicrobial, anti-influenza, and anti-orthopoxvirus agents [32–35]. These modified nucleosides recruit the potential of simultaneous recognition of two heterocyclic bases in a bisubstrate type of interaction. Alternatively, "double-headed" nucleosides may interact with nucleic acids using one base for Watson–Crick pairing and the second one as the intercalator stabilizing the base pair. Indeed, the latter possibility has been realized in a recent study [36,37] (Fig. 2).

1.1. Chemistry

The synthetic approach to acyclic pyrimidine nucleoside analogues **4a**–**7b** involved first the preparation of the known 1-[(2-acetoxyethoxy)methyl]-thymine (**3a**), followed by its oxidation to 1-[(2-acetoxyethoxy)methyl]-5-formyluracil (**4a**) followed by acid-catalyzed reaction with CH_3OH and deprotection to give the previously unknown 1-(2-hydroxyethoxymethyl)-5-(dimethoxymethyl)uracil (**5b**). Alternatively, 1-[(2-acetoxyethoxy)methyl]-5-formyluracil (**4a**) itself could be deacetylated to provide 1-(2-hydroxyethoxymethyl)-5-formyluracil (**4b**), also previously unreported. This latter compound was the basis for the preparation of 1-(2-hydroxyethoxymethyl)-5-(2,2-dicyanovinyl)uracil (**6b**) directly from malononitrile. Likewise reaction of malononitrile with 1-[(2-acetoxyethoxy)methyl]-5-formyluracil (**4a**) yielded 1-[(2-acetoxyethoxy)methyl]-5–(2,2-dicyanovinyl)uracil



3a: $R_1 = CH_3$; R_2 = Ac
3b: $R_1 = CH_3$; R_2 = H
4a: R_1 = CHO; R_2 = Ac
4b: R_1 = CHO; R_2 = H
5a: $R_1 = CH(OCH_3)_2$; R_2 = Ac
5b: $R_1 = CH(OCH_3)_2$; R_2 = H
6a: $R_1 = CH{=}C(CN)_2$; R_2 = Ac
6b: $R_1 = CH{=}C(CN)_2$; R_2 = H
7a: R_1 =  ; R_2 = Ac

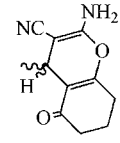
7b: R_1 =  ; R_2 = H

Fig. 2. Acyclic nucleoside analogues prepared to be evaluated as potential antiviral agents.

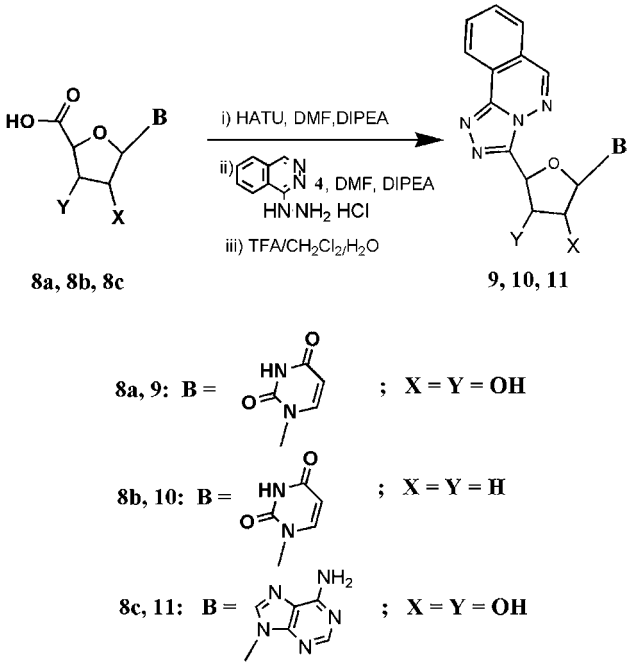


Fig. 3. Synthetic approach to "double-headed" nucleoside analogues.

(**6a**). Similarly, reaction 1-[(2-acetoxyethoxy)methyl]-5-formyluracil (**4a**) with malononitrile and 1,3-cyclohexanedione gave rise to 1-[(2-acetoxyethoxy)methyl]-5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4H-chromen-4-yl)uracil (**7a**) and reaction of malononitrile and 1,3-cyclohexanedione with 1-[(2-hydroxyethoxy)methyl]-5-formyluracil (**4b**) gave 1-[(2-hydroxyethoxy)methyl]-5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4H-chromen-4-yl)-uracil (**7b**).

Synthesis of the "double-headed" nucleosides (**9**, **10**, and **11**) was based on the utilization of carboxylic acid nucleosides **8a**–**8c** at carbon 5′ as the synthon for the second heterocyclic base. These uronic acid congeners were generated by application of reported procedures [38]. Reaction of **8a**, **8b**, or **8c** with phthalazin-1-yl-hydrazine hydrochloride in dimethylformamide in the presence of diisopropylethylamine (DIEA) as a base and 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU) as coupling reagent at 0–3° followed by treatment of the residual after the work up gave the triazolophthalazine derivatives **9**, **10**, and **11**, respectively, in acceptable yields (Fig. 3).

1.2. Antiviral activities

Synthetic products were evaluated for antiviral activity against HIV-1, HIV-2, vaccinia virus, cowpox virus, herpes simplex viruses types 1 and 2, parainfluenza virus type 3, Punta Toro virus, vescicular stomatitis virus, respiratory syncytial virus, Coxsackie B4 virus, reovirus type 1, and Sindbis virus (Tables 1 and 2).

Table 1
Antiviral activities of compounds **3a**–**11** against selected RNA viruses in Vero, HeLa, and HEL cells

Compound	EC_{50}[a]						
	Parainfluenza virus type 3 (Vero)	Reovirus type 1 (Vero)	Sindbis virus (Vero)	Punta Toro virus (Vero)	Vescicular stomatitis virus (HEL/HeLa)	Coxsackie B4 virus (HeLa/Vero)	Respiratory syncytial virus (HeLa)
3a	>200	>200	>200	>200	>200	>200	>200
3b	>200	>200	>200	>200	>200	>200	>200
4a	>40	>40	>200	>40	>200	>200	>200
4b	>40	>40	>200	>40	>200	>200	>200
5a	>200	>200	>200	>200	>200	>200	>200
5b	>200	>200	>200	>200	>200	>200	>200
6a	>100	>100	>100	>100	>100	>100	>100
6b	>100	>100	>100	>100	>100	>100	>100
7a	>100	>100	>100	>100	>100	>100	>100
7b	>100	>100	>100	>100	>100	>100	>100
9	>100	>100	>100	>100	>100	>100	>100
10	>100	>100	>100	>100	>100	>100	>100
11	>100	>100	>100	>100	>100	>100	>100

[a] Concentration (μM) required to effect a 50% reduction in virus-induced cytopathogenicity in either Vero cells, HEL or HeLa cells, as indicated.

Table 2
Antiviral activities of compounds **3a**–**11** against HIV-1, HIV-2, vaccinia virus (VV), cowpox virus (CV), and herpes viruses

Compound	EC_{50}						
	HIV-1 (CEM)	HIV-2 (CEM)	VV (HEL)	VV (HSF)	CV (HSF)	HSV-1 (KOS) (HEL)	HSV-2 (G) (HEL)
3a	>250	>250	>200	>300	>300	>200	>200
3b	>250	>250	>200	>300	>300	>200	>200
4a	>250	>250	>200	>300	>300	>200	>200
4b	>250	>250	>200	>300	>300	>200	>200
5a	>250	>250	>200	>300	>300	>200	>200
5b	>250	>250	>200	>300	>300	>200	>200
6a	>50	>50	100	>60	>60	>100	100
6b	>50	>50	>100	>300	>300	>100	>100
7a	>250	>250	>100	>300	>300	>100	>100
7b	>250	>250	>100	>300	>300	>100	>100
9	>250	>250	>100	>300	>300	>100	>100
11	>250	>250	>100	>300	>300	>100	>100

Concentration (μM) required to effect a 50% reduction in virus-induced cytopathogenicity in either CEM cells, HEL or HSF cells, as indicated. Under the conditions employed above, both Acyclovir and Ganciclovir displayed and a submicromolar EC_{50} against HSV-1 and HSV-2 and Brivudin showed an EC_{50} of 12 μM against vaccinia virus assayed in HEL cells. Cowpox virus (CV) and vaccinia virus (VV) showed EC_{50}s in the 10 μM range when they were assayed in HSF cells.

2. Discussion

Although the parent nucleosides **2a**, **2b**, and **2c** all possess potent in vitro antiviral activity [16–19] against both of the orthopoxviruses vaccinia virus and cowpox virus, their acyclic analogues, **5b**, **6b**, and **7b**, were devoid of activity. This may be a consequence of the substrate specificity of the orthopoxviral thymidine kinase. Recently, we have presented evidence that at least one of the prototype antivirals, **2c**, depends upon the viral thymidine kinase for antiviral activity [19]. Moreover, the active analogue **2c** is a good substrate for vaccinia thymidine kinase [19]. Even though these studies have indicated that orthopoxviral thymidine kinase may be much more promiscuous than previously believed, this promiscuity may not extend to acceptance of such hypermodification of the sugar domain as embodied in the structures of **5b**, **6b**, and **7b**. In support of this hypothesis was the earlier observation that acyclovir is not phosphorylated by vaccinia thymidine kinase [39].

This line of reasoning probably also explains the inactivity of **5b**, **6b**, and **7b** against the herpes simplex viruses evaluated. We have found that compounds such as **2c** are antivirally active against HSV-1 and varicella zoster virus, again by virtue of their phosphorylation by the respective viral thymidine kinases (Prichard et al., data not shown). Thus although the relatively promiscuous herpes simplex virus thymidine kinase readily phosphorylates the acyclic nucleoside analogue acyclovir, it may not accept the hypermodification represented by **7b** and its congeners.

The inactivity of the ''double-headed'' nucleosides (**9**–**11**), at least against the orthopoxviruses and herpes viruses, is also most probably connected to the inability of these compounds to be phosphorylated because of the missing 5′-hydroxyl moiety.

While overall, this study revealed no intriguing antiviral activities, it does define more clearly the boundaries in a future search for efficacious orthopoxvirus countermeasures.

3. Experimental section

Melting points were recorded with a Barnstead 1201D electrothermal melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer. $CDCl_3$, CD_3OD or DMSO-d_6 was used as the NMR solvent for different compounds. The chemical shifts of the deuterated solvent served as internal standard. The mass spectra were performed on a HP 1100 MSD spectrometer at the HT Laboratories, San Diego. The HRMS (High Resolution Mass Spectra) were performed on a JEOL HX 110A spectrometer at the Department of Chemistry, University of Arizona. Silica gel column chromatography was conducted with Sigma–Aldrich silica gel (70 ~ 230 mesh). The preparation of (2-acetoxyethoxy)methyl bromide from 1,3-dioxolane and acetyl bromide was according to the procedure of Robins and Hatfield [40].

3.1. 1-[(2-Acetoxyethoxy)methyl]-thymine (**3a**)

A suspension of 0.63 g (5 mmol) of thymine in 15 mL of anhydrous CH_2Cl_2 was added 3 mL (12 mmol) of *N*,*O*-bis(trimethylsilyl)acetamide (BSA) under nitrogen. The mixture was stirred at room temperature. After the solution became clear, (2-acetoxyethoxy)methyl bromide (1.0 g, 5 mmol) was added dropwise. After 2 h, the reaction liquid was poured slowly into a mixture of cold saturated aqueous $NaHCO_3$ (25 mL) and $CHCl_3$ (50 mL). The resulting emulsion was separated by filtration. The aqueous layer was extracted with EtOAc (3 × 25 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. After purification through a silica gel column with $CHCl_3$—CH_3COCH_3 (3:1) as the eluant, 1-[(2-acetoxyethoxy)methyl]-thymine (**3a**) was obtained (0.94 g, 3.88 mmol, colorless solid, yield 78%): mp 115–117 °C (Lit. [34] 123–125 °C); ^{1}H NMR ($CDCl_3$) δ: 1.89 (s, 3H, CH_3), 2.02 (s, 3H, CH_3CO), 3.75(A_2B_2-t, $J = 4.7$ Hz, 2H, $AcOCH_2CH_2O$), 4.16 (A_2B_2-t, $J = 4.7$ Hz, 2H, $AcOCH_2CH_2C$), 5.14 (s, 2H, NCH_2O), 7.13 (s, 1H, C_6H), 9.92 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 12.5, 21.0, 63.2, 67.7, 76.7, 112.1, 139.1, 151.5, 164.1, 171.0; MS (ES) *m*/*e*: 241 $[M-H]^-$, 265 $[M+Na]^+$.

3.2. 1-(2-Hydroxyethoxymethyl)-thymine (**3b**)

NaOMe in MeOH (3 mL, 0.5 N) was added to a solution of **3a** (0.3 g, 1.24 mmol) in 10 mL of anhydrous MeOH. The mixture was stirred at room temperature. After the reaction was complete (TLC), Amberlite IR-120 acid resin was added to adjust the pH value to 7. The resin then was filtrated, and the solvent was evaporated under reduced pressure to provide a colorless solid. After purification by a silica gel column with $CHCl_3$—MeOH (5:1) as the eluant, the product 1-(2-hydroxyethoxymethyl)-thymine (**3b**) was obtained as colorless crystals in a yield of 93% (0.23 g, 1.15 mmol): mp 140–141 °C (Lit. [40] 150.5–152 °C); ^{1}H NMR (DMSO-d_6) δ: 1.75 (d, $J = 1.2$ Hz, 3H, CH_3), 3.45–3.46 (A_2B_2 m, 4H, OCH_2CH_2O), 4.61–4.64 (m, 1H, OH), 5.04 (s, 2H, NCH_2O), 7.55 (d, $J = 1.2$ Hz, 1H, C_6H), 11.28 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 12.5, 60.7, 71.1, 77.0, 109.8, 141.2, 151.8, 164.9; MS (ES) *m*/*e*: 199 $[M-H]^-$, 223 $[M+Na]^+$.

3.3. 1-[(2-Acetoxyethoxy)methyl]-5-formyluracil (**4a**)

A CH_3CN solution (10 mL) containing 1-[(2-acetoxyethoxy)methyl]thymine (**3a**) (0.61 g, 2.5 mmol) and 2,6-lutidine (1 mL) was added to a solution of $K_2S_2O_8$ (1.35 g, 5 mmol) and $CuSO_4{\cdot}5H_2O$ (0.25 g, 1 mmol) in 10 mL H_2O. The mixture was stirred at 65° for 2 h. After completion, the mixture was concentrated to half of the initial volume and the remaining solution was extracted with EtOAc (3×25 mL). The organic layers were combined and washed with H_2O. The aqueous layer was back-extracted with $CHCl_3$. All the organic liquids were combined, dried over anhydrous Na_2SO_4, and then concentrated. The residue was purified through a silica gel column chromatography with EtOAc–hexane (2:1) as eluant. The fractions were collected, concentrated, and the residue recrystallized from EtOAc to give 1-[(2-acetoxyethoxy)methyl]-5-formyluracil (**4a**) as colorless crystals (0.20 g, 0.78 mmol, 31%): mp 115–116 °C; ^{1}H NMR ($CDCl_3$) δ: 2.07 (s, 3H, CH_3CO), 3.81–3.84 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 4.21–4.23 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 5.29 (s, 2H, NCH_2O), 8.23 (s, 1H, C_6H), 9.25 (s, 1H, NH), 10.02 (s, 1H, CHO); ^{13}C NMR ($CDCl_3$) δ: 21.0, 63.0, 68.6, 78.1, 112.1, 148.3, 150.1, 162.0, 171.0, 186.1; MS (ES) *m*/*e*: 255 $[M-H]^-$, 279 $[M+Na]^+$; HRMS (FAB) Calcd for $C_{10}H_{13}N_2O_6$: 257.0773 $[M+H]^+$. Found: 257.0785.

3.4. 1-[(2-Acetoxyethoxy)methyl]-5-(dimethoxymethyl)uracil (**5a**)

1-[(2-Acetoxyethoxy)methyl]-5-formyluracil (**4a**) (0.37 g, 1.45 mmol) was dissolved in anhydrous methanol (15 mL), and this solution refluxed with stirring in the presence of washed dry Amberlite IR-120 acid resin (0.15 g) for 40 min. The mixture was filtered to remove the resin and the filtrate was concentrated to give a colorless solid. The solid was washed with anhydrous methanol and anhydrous ethyl ether and the resulting colorless solid, 1-[(2-acetoxyethoxy)methyl]-5-(dimethoxymethyl)uracil (**5a**), was obtained in a yield of 98% (0.43 g, 1.42 mmol): mp 113–114 °C; ^{1}H NMR (CD_3OD) δ: 2.02 (s, 3H, CH_3CO), 3.36 (s, 6H, $(OCH_3)_2$), 3.77–3.79 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 4.17–4.19 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 5.22 (s, 2H, NCH_2O), 5.28 (d, $J = 0.8$ Hz, 1H, $CH(OCH_3)_2$), 7.70 (d, $J = 0.8$ Hz, 1H, C_6H); ^{13}C NMR (CD_3OD) δ: 19.5, 52.8, 63.3, 67.4, 77.1, 98.2, 111.5, 142.9, 151.5, 163.4, 171.5; MS (ES) *m*/*e*: 301 $[M-H]^-$, 325 $[M+Na]^+$; HRMS (ESI) Calcd for $C_{12}H_{18}N_2O_7Na$: 325.1012 $[M+Na]^+$. Found: 325.1002.

3.5. 1-(2-Hydroxyethoxymethyl)-5-(dimethoxymethyl)uracil (**5b**)

A NH_3/MeOH solution (2 mL, 7 N) was added to a solution of 1-[(2-acetoxyethoxy)methyl]-5-(dimethoxymethyl)-uracil (**5a**) (0.30 g, 1 mmol) in anhydrous methanol (8 mL). The mixture was stirred in an ice bath for 3 h, then at ambient temperature for 5 h. The solvent then was removed under high vacuum. The residue was purified through a silica gel column with $CHCl_3$—MeOH (6:1) as the eluant to give the corresponding product 1-(2-hydroxyethoxymethyl)-5-(dimethoxymethyl)uracil (**5b**) in a yield of 96% (0.25 g, 0.96 mmol, light yellow solid): mp 92–94 °C; ^{1}H NMR (CD_3OD) δ: 3.35 (s, 6H, $(OCH_3)_2$), 3.61–3.66 (A_2B_2 m, 4H, $AcOCH_2CH_2O$), 5.22 (s, 2H, NCH_2O), 5.28 (d, $J = 0.8$ Hz, 1H, $CH(OCH_3)_2$), 7.72 (d, $J = 0.8$ Hz, 1H, C_6H); ^{13}C NMR (CD_3OD) δ: 52.8, 60.8, 70.9, 77.3, 98.1, 111.3, 143.1, 151.5, 163.4; MS (ES) *m*/*e*: 259 $[M-H]^-$, 283 $[M+Na]^+$; HRMS (ESI) Calcd for $C_{10}H_{16}N_2O_6Na$: 283.0906 $[M+Na]^+$. Found: 283.0904.

3.6. 1-(2-Hydroxyethoxymethyl)-5-formyluracil (**4b**)

1-(2-Hydroxyethoxymethyl)-5-(dimethoxymethyl)uracil (**5b**) (0.26 g, 1 mmol) was added to a solution of 15 mL acetic acid and 3 mL H_2O. The mixture was stirred at 50° overnight. The solvent was evaporated under reduced pressure, and the residue was purified through a silica gel column with $CHCl_3$—MeOH (6:1) as eluant. The fractions were collected, concentrated, and the residue was recrystallized from EtOH to give 1-(2-hydroxyethoxymethyl)-5-formyluracil (**4b**) as light yellow solid (0.21 g, 0.98 mmol 98%): mp 138–139.5 °C; ^{1}H NMR (DMSO-d_6) δ: 3.44–3.48 (A_2B_2 m, 2H, OCH_2CH_2OH), 3.52–3.54 (A_2B_2 m, 2H, OCH_2CH_2OH), 4.66 (t, $J = 5.2$ Hz, 1H, OH), 5.22 (s, 2H, NCH_2O), 8.49 (s, 1H, C_6H), 9.77 (s, 1H, CHO), 11.76 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 60.7, 71.7, 78.3, 111.4, 150.8, 151.8, 162.7, 187.1; MS (ES) *m/e* 213 $[M-H]^-$, 237 $[M+Na]^+$; HRMS (FAB) Calcd for $C_8H_{11}N_2O_5$: 215.0668 $[M+H]^+$. Found: 215.0659.

3.7. 1-[(2-Acetoxyethoxy)methyl]-5-(2,2-dicyanovinyl)uracil (**6a**)

Malononitrile (92 mg, 1.4 mmol) and 1-[(2-acetoxyethoxy)methyl]-5-formyluracil (**4a**) (179 mg, 0.7 mmol) were dissolved in anhydrous EtOH (8 mL). The mixture was stirred at 50° overnight. The resulting reaction mixture was concentrated, and the residue was purified through silica gel column chromatography with EtOAc–hexane (2:1) as eluant. The fractions were collected, concentrated, and the residue was recrystallized from EtOH to give 1-[(2-acetoxyethoxy)methyl]-5-(2, 2-dicyanovinyl)uracil (**6a**) as pale yellow crystals (157 mg, 0.52 mmol, 74%): mp 137–139 °C; ^{1}H NMR (CD_3OD) δ: 2.02 (s, 3H, CH_3CO), 3.83–3.85 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 4.19–4.22 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 5.31 (s, 2H, NCH_2O), 7.97 (s, 1H, CH=), 8.76 (s, 1H, C_6H); ^{13}C NMR (CD_3OD) δ: 19.5, 63.2, 68.1, 78.3, 79.5, 107.7, 113.0, 113.8, 148.5, 150.0, 151.4, 161.3, 171.5; MS (ES) *m/e*: 303 $[M-H]^-$; HRMS (FAB) Calcd for $C_{13}H_{13}N_4O_5$: 305.0886 $[M+H]^+$. Found: 305.0890.

3.8. 1-(2-Hydroxyethoxymethyl)-5-(2, 2-dicyanovinyl)uracil (**6b**)

Malononitrile (79 mg, 1.2 mmol) and 1-(2-hydroxyethoxymethyl)-5-formyluracil (**4b**) (128.4 mg, 0.6 mmol) were dissolved in anhydrous EtOH (10 mL). The mixture was stirred at room temperature overnight. The resulting reaction mixture was concentrated, and the residue was purified through silica gel column chromatography with $CHCl_3$—MeOH (6:1) as eluant. The fractions were collected, concentrated, and the residue was recrystallized from EtOH to give 1-(2-hydroxyethoxymethyl)-5-(2,2-dicyanovinyl)uracil (**6b**) as a yellow solid (102 mg, 0.39 mmol, 65%): mp 124–125 °C; ^{1}H NMR (CD_3OD) δ: 3.65–3.71 (A_2B_2 m, 4H, OCH_2CH_2O), 5.32 (s, 2H, NCH_2O), 7.96 (s, 1H, CH=), 8.78 (s, 1H, C_6H); ^{13}C NMR (CD_3OD) δ: 60.7, 71.7, 78.5, 79.2, 107.5, 113.1, 113.9, 148.7, 150.0, 151.4, 161.4; MS (ES) *m/e*: 261 $[M-H]^-$, 285 $[M+Na]^+$; HRMS (FAB) Calcd for $C_{11}H_{11}N_4O_4$: 263.0780 $[M+H]^+$. Found: 263.0790.

3.9. 1-[(2-Acetoxyethoxy)methyl]-5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4H-chromen-4-yl)uracil (**7a**)

1-[(2-acetoxyethoxy)methyl]-5-formyluracil (**4a**) (102 mg, 0.4 mmol), malononitrile (33 mg, 0.5 mmol), and 1,3-cyclohexanedione (57 mg, 0.5 mmol) were dissolved in

anhydrous ethanol (8 mL). The mixture was stirred at 50 ° overnight. The colorless precipitate that formed was filtered off and rinsed with ethanol and ethyl ether to give the product 1-[(2-acetoxy-ethoxy)-methyl]-5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4H-chromen-4-yl)uracil (**7a**) (100 mg). The filtrate was concentrated and the residue was purified through silica gel column chromatography with $CHCl_3$—MeOH (5:1) as eluant to yield an additional 35 mg quantity of compound **7a**. Thus, the product **7a** was obtained in a total yield of 81% (135 mg, 0.32 mmol): mp 202–204 °C; ^{1}H NMR (DMSO-d_6) δ: 1.82–1.95 (m, 2H, CH_2), 1.98 (s, 3H, CH_3CO), 2.20–2.32 (m, 2H, CH_2), 2.45–2.52 (m, 2H, CH_2), 3.66–3.68 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 4.01(s, 1H, CH), 4.05–4.08 (A_2B_2 m, 2H, $AcOCH_2CH_2O$), 5.08 (s, 2H, NCH_2O), 6.89 (s, 2H, NH_2), 7.58 (s, 1H, C_6H), 11.3 (s, 1H, NH); ^{13}C NMR (CD_3OD) δ: 20.5, 21.3, 27.3, 29.4, 36.9, 55.9, 63.6, 67.5, 77.2, 111.8, 115.7, 120.4, 142.5, 151.5, 160.1, 162.8, 166.1, 171.0, 196.6; MS (ES) *m/e*: 415 $[M-H]^-$, 439 $[M+Na]^+$; HRMS (FAB) Calcd for $C_{19}H_{21}N_4O_7$: 417.1410 $[M+H]^+$. Found: 417.1410.

*3.10. 1-[(2-Hydroxyethoxy)methyl]-5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro-4H-chromen-4-yl)uracil (**7b**)*

1-[(2-hydroxyethoxy)methyl]-5-formyluracil (**4b**) (107 mg, 0.5 mmol), malononitrile (40 mg, 0.6 mmol) and 1,3-cyclohexanedione (67 mg, 0.6 mmol) were dissolved in anhydrous ethanol (8 mL). The mixture was stirred at 50° overnight. The colorless precipitate that formed was filtered off and rinsed with ethanol and ethyl ether to give the product 1-[(2-hydroxyethoxy)methyl]-5-(2-amino-3-cyano-5-oxo-5,6,7,8-tetrahydro- 4H-chromen-4-yl)uracil (**7b**) (110 mg). The filtrate was concentrated and the residue was purified through silica gel column chromatography with $CHCl_3$—MeOH (5:1) as eluant to give an additional 45 mg quantity of compound **7b**. Thus, the product **7b** was obtained in a total yield of 83% (155 mg, 0.41 mmol): mp 208–209.5 °C; ^{1}H NMR (DMSO-d_6) δ: 1.82–1.95 (m, 2H, CH_2), 2.20–2.28 (m, 2H, CH_2), 2.45–2.52 (m, 2H, CH_2), 3.43–3.49 (A_2B_2 m, 4H, $AcOCH_2CH_2O$), 4.02(s, 1H, CH), 4.63–4.66 (m, 1H, OH), 5.07 (s, 2H, NCH_2O), 6.88 (s, 2H, NH_2), 7.56 (s, 1H, C_6H), 11.2 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 20.5, 27.3, 29.3, 37.0, 56.0, 60.7, 71.2, 77.4, 111.8, 115.6, 120.4, 142.6, 151.4, 160.1, 162.9, 166.0, 196.6; MS (ES) *m/e*: 373 $[M-H]^-$, 397 $[M+Na]^+$; HRMS (FAB) Calcd for $C_{17}H_{19}N_4O_6$: 375.1304 $[M+H]^+$. Found: 375.1299.

3.11. General procedure for coupling of carboxylic acid nucleosides with hydralazine hydrochloride

Step 1. A mixture of carboxylic acid nucleoside (1 mmol), HATU (2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methan-aminium, 0.38 g, 1 mmol) and DIPEA (diisopropylethylamine) (0.34 mL, 2 mmol) was stirred at 0 °C for 3 min. [preactivation period] in 4 mL anhydrous DMF (dimethylformamide). A solution of hydralazine.HCl (0.186 g), and DIPEA (0.18 mL, mmol) in DMF (4 mL) was added to this solution at 0°. The reaction mixture was stirred at 0 °C for 3 h and left overnight at room temperature. The reaction mixture was diluted with ethyl acetate (80 mL), and the mixture was washed with 5% aqueous citric acid solution (2×15 mL), saturated sodium bicarbonate solution (2×15 mL) and saturated sodium chloride solution (2×15 mL). The organic layer was dried over anhydrous sodium sulphate, filtered and the solvent was removed in vacuo.

Step 2. The residual solid was treated with methylene chloride (5 mL), TFA (5 mL) and water (0.2 mL). The mixture was stirred at room temperature for 2 h. The solvent was evaporated in vacuo and the residue was subjected to column chromatography on silica gel, eluting with $CHCl_3$—MeOH (4:1). The following compounds were obtained after solvent evaporation *in vacuo*.

From compound **8a**, compound **9**, mp 175–177 °C (dec) was obtained in a yield of 20%: ^{1}H NMR (DMSO-d_6) δ: 4.52 (''dd'', 1H, H-2′), 4.56 (''dd'', 1H, H-3′), 5.53 (d, $J = 3.2$ Hz, 1H, H-4′), 5.73 (d, 1H, H-5), 5.75 (br s, 2H, 2OH, D_2O exchangeable), 6.10 (d, $J = 6$ Hz, 1 H, H-1′), 7.94, 8.09, 8.22, 8.50, 9.14 (t, m, d, d, s, 6H, ArH and H-6), 11.37 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 73.32, 73.93, 76.10, 88.80, 103.06, 122.89, 123.22, 123.88, 129.92, 132.06, 135.23, 141.22, 143.52, 149.08, 149.56, 151.51, 163.71; HRMS–FAB$^+$ Calcd for $C_{17}H_{15}N_6O_5$: 383.1113 $[M+H]^+$. Found: 383.1104.

From compound **8b**, compound **10**, mp 231–233 °C was obtained in a yield of 19%: ^{1}H NMR (DMSO-d_6) δ: 2.56–2.62 (m, 4H, 2H-2′, and 2H-3′), 5.64 (''d'', 1H, H-5), 5.67 (''dd'', 1H, H-4′), 6.24 (''dd'', 1H, H-1′), 6.98, 8.08, 8.23, 8.52, 9.14 (m, t, d, d, s, 6H, ArH, and H-6), 11.31 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 31.67, 31.95, 72.27, 86.61, 102.32, 122.91, 123.27, 123.87, 129.89, 132.03, 135.19,, 141.16, 143.63, 149.40, 149.98, 151.18, 163.83; HRMS–FAB$^+$ Calcd for $C_{17}H_{15}N_6O_3$: 351.1206 $[M+H]^+$. Found: 351.1216 .

From compound **8c**, compound **11**, mp 198–200 °C, was obtained in a yield of 25%; ^{1}H NMR (DMSO-d_6) δ: 4.90 (''dd'', 1H, H-3′), 4.97 (''dd'', 1H, H-2′), 5.60 (d, $J = 4$ Hz, 1H, H-4′), 5.85, 5.87 (2d overlapped ~t, 2H, 2OH, D_2O exchangeable), 6.19 (d, 1H, $J = 5.6$ Hz, H-1′), 7.27 (br s, 2H, NH_2, D_2O exchangeable), 7.93, 8.06, 8.11, 8.21, 8.49, 8.51, 9.10 (t, t, s, d, s, d, s, 7H, ArH); ^{13}C NMR (DMSO-d_6) δ: 69.37, 70.90, 72.29, 84.06, 115.52, 118.98, 119.28, 119.92, 125.96, 128.09, 131.27, 135.70, 139.66, 145.05, 145.46, 146.46, 149.55, 152.79; HRMS–FAB$^+$ Calcd for $C_{18}H_{16}N_9O_3$: 406.1376 $[M+H]^+$. Found: 406.1373.

4. Antiviral evaluation procedures

These were carried out according to previously published procedures [16–19,41] pursuant to the footnotes of Tables 1 and 2.

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