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Journal:	*New Journal of Chemistry*
Manuscript ID	NJ-ART-08-2020-004154.R1
Article Type:	Paper
Date Submitted by the Author:	16-Oct-2020
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2-Hydroxyimino-6-aza-pyrimidine Nucleosides: Synthesis, DFT Calculations, and Antiviral Evaluations

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Abstract.

The global public health concerns and economic impact caused by emerged outbreaks of RNA viruses call for the search for new direct acting antiviral agents. Herein, we describe the synthesis, DFT calculations, and antiviral evaluation of a series of novel 2-hydroxyimino-6-aza-pyrimidine ribonucleosides. DFT//B3LYP/6-311+G** calculations of the tautomeric distributions of the 2-hydroxyimino nucleosides **7**, **8**, and **9** in aqueous environment indicate a predominance of the canonical 2-(*E*)-hydroxyimino structure, where the hydroxyl group points away from the sugar moiety. Conformer distribution of the latter geometrical isomers of **7**, **8**, and **9** support the formation of five membered rings *via* hydrogen bonding between the (*E*)-C^2=N-O-H moiety and N^3-H of **7** and **8** and between (*E*)-C^2=N-O-H and N^3 with **9**, creating purine shaped nucleosides with the glycosidic linkage at the pyrimidine ring. The newly synthesized nucleosides were screened against RNA viral panel, of which moderate antiviral activity was observed against Zika virus (ZIKV) and human respiratory syncytial virus (HRSV). 6-Aza-2-hydroxylimino-5-methyluridine derivative **18** showed activity against ZIKV (EC_{50} 3.2 μM), while its peracetylated derivative **19** showed activity against HRSV (EC_{50} 5.2 μM). The corresponding 4-thiono-2-hydroxylimino derivative **8** showed activity against HRSV (EC_{50} 6.1 μM) and against ZIKA (EC50 2.4 μM). This study shows that the 6-aza-2-hydroxyimino-5-methyluracil derived nucleosides can be further optimized to provide potent antiviral agents.

Keywords: 6-Aza-5-methyluridine, Nucleosides, anti-HRSV, Anti-MERS-CoV, anti-ZIKV, Hepatitis C virus.

Introduction

Recent outbreaks of RNA viruses such as Zika virus (ZIKV)[1, 2], Middle East respiratory syndrome coronavirus (MERS-CoV)[3], Influenza A viruses (IAVs) of human, avian, and swine origins are causing global public health concerns and associated with large economic impact. ZIKV is a positive-stranded RNA (+RNA) flavivirus[4, 5], transmitted by mosquitoes and sexually as well [6]. ZIKV is associated with severe neural disorders, such as microcephaly in fetuses and newborns and Guillain-Barr's syndrome in adults. MERS-CoV can cause serious respiratory diseases with 38% fatality rate of infected patients [7-9]. Human respiratory syncytial virus (HRSV), a member of *Paramyxoviridae* family, is a single stranded RNA (-RNA) virus [10]. HRSV infection causes acute upper and lower respiratory tract infection in infants, children, elderly, and immune compromised adults, with significant morbidity and mortality. Severe HRSV infection causes around 160K deaths per year in children under 5 years old worldwide [11, 12]. Ribavirin (**1**), a broad-spectrum antiviral agent, is the only small molecule approved for the treatment of HRSV infection (Fig. 1). Inhibiting inosine monophosphate dehydrogenase (IMPDH) and inducing viral mutagenesis are the most two important mechanisms by which ribavirin induces its antiviral activity [13-15]. However, toxicity liabilities and limited efficacy undermine the clinical use of ribavirin [16, 17]. Also, there are no effective drugs or vaccines available for the treatment or prevention of ZIKV and MERS-CoV, infections. These unmet medical needs call for the search

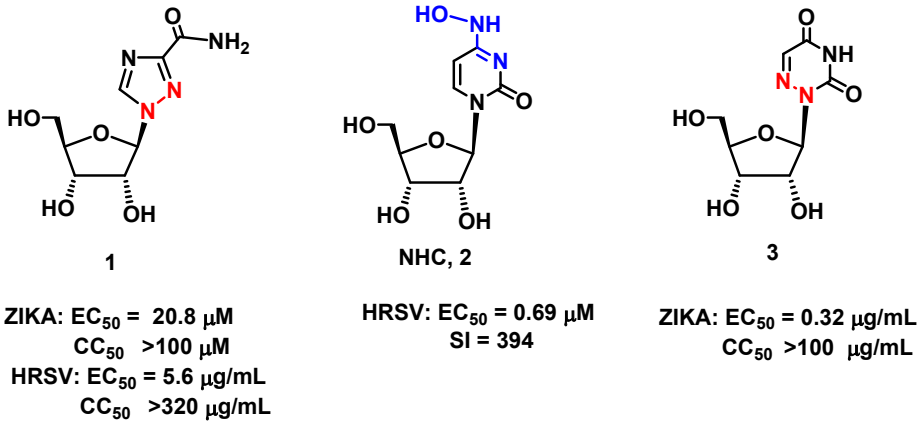


Fig. 1. Structures of base modified nucleosides with activity against Zika and RSV viruses.

for new agents that combat these viruses. N^4-Hydroxycytidine (NHC, **2**), a cytidine/uridine analogue, has been reported to inhibit HRSV, influenza B virus, and Influenza A viruses (IAVs) of human, avian, and swine origins, and severe acute respiratory syndrome-associated coronavirus (SARS-CoV) [18] (Fig. 1). NHC has shown inhibitory activity against Ebola virus replication [19], Norovirus [20], bovine diarrhea virus (BVDV) and Hepatitis C (HCV) virus [21], anti-Venezuelan equine encephalitis virus (VEEV) [22]. Recently, NHC has been reported to have activity both in vitro and im mice against SARS-CoV-2 [23]. The antiviral activity of NHC relates primarily to the incorporation of NHC-triphosphate derivative into viral genome in place of uridine, in which case, NHC behaves both as cytosine and uracil, resulting in high mutation frequencies and, ultimately, error catastrophe.[24]

Azapyrimidine nucleosides with triazine as a nucleobase are associated with antitumor [25] and antiviral activities. 6-Azacytidine (6-azaCyd) has demonstrated anti-human adenoviral activity *in vitro* and *in vivo* [26-31]. 6-Azauridine (6-azaUrd, **3**), a pyrimidine synthesis inhibitor, has been reported to have anti-ZIKV [32], anti-Lassa, anti-Ebola [33], and anti-Chikungunya (CHIKV) viral activity [34]. Furthermore, 6-azaUrd, 6-azauridine triacetate, and 2-thio-6-azaurdine were also reported to have anti-West Nile virus (New York isolate) activity [35].

In this study, we envisioned that replacing the C^2=O moiety of 6-aza-pyrimidin-1-yl nucleobase with C^2=N-OH and C^2=S would maintain the geometrical C^2 exocyclic double bond required for enzyme recognition, while altering the steric and electronic effects on the ring. Herein, we wish to report on the synthesis of 5-methyl-6-azapyrimidine nucleosides **4-9** (Fig. 2), Density Functional Theory (DFT) calculations of their tautomeric and conformer distributions, and the evaluation of their activity and that of their intermediates **11-19** against RNA viral panel.

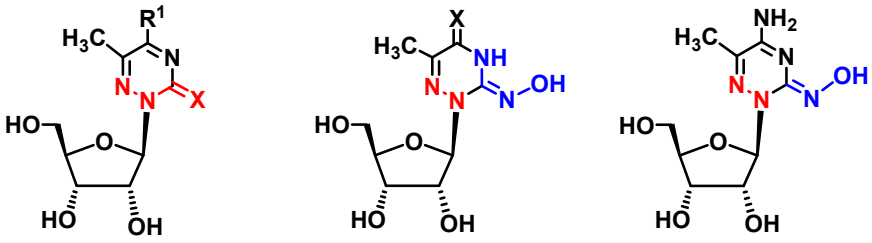


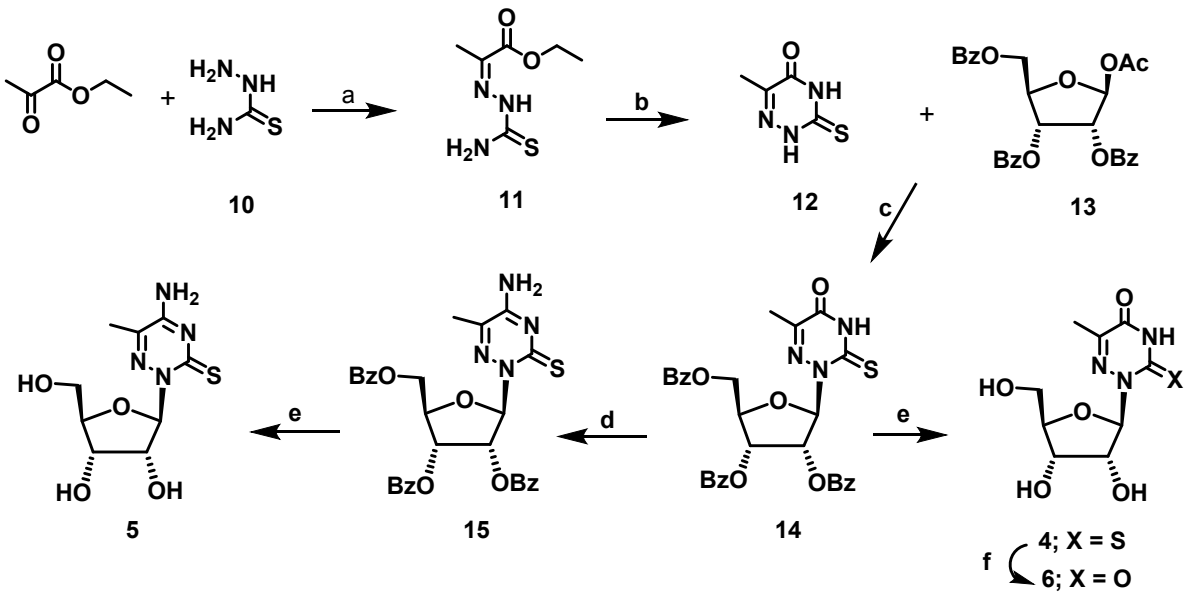
Fig. 2. Known 6-azanucleosides 4, 5, 7 and additional nucleosides 6, 8, 9 investigated in this study.

Results and Discussion

Chemistry

We have previously reported the synthesis of 2-selenothymidine and 2-selenouridine derivatives *via* NaSeH nucleophilic substitution of C^2-methylthiothymidine and uridine derivatives [36, 37]. We have adopted this strategy for modifying the C^2 position of 6-aza-5-methyluridine with =NOH moiety. The precursor for the corresponding 2-methylthio derivative, 2-thio-6-azauridine derivative have been synthesized *via* Vorbrüggen-type glycosylation [38] of 6-aza-5-methyl-2-thiouracil (**12**) with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**13**) [39]. The synthesis of **12** is outlined in scheme 1. Reaction of ethyl pyruvate and thiosemicarbazide provided the corresponding carbazone derivative **11** in quantitative yield. Cyclization of the carbazone derivative **11** under basic conditions (NaOH/EtOH/reflux) [40], however did not provide the cyclized product, 2-thio-6-azathymine (**12**) in a satisfactory yield. Conducting the reaction in the presence of anhydrous NaOAc/AcOH under fusion conditions gave **12** in 87% yield (Table 1, entry 1). Performing the reaction using NaOAc/AcOH in absolute EtOH gave **12** in quantitative yield as a pale-yellow crystals (table 1, entry 2, 3).

Scheme 1[a]



[a]Reagents and conditions. a) EtOH, reflux, 2 h; b) NaOAc. AcOH, EtOH, reflux 2.5 h; c) i) BSA, DCE, rt, 30 min; ii) $SnCl_4$, -10, 3 h, then DMSO, rt, 1 h; d) TBSCl, DMAP, CH_3CN., TEA, 12 h, then NH_3 gas, 0 ºC, 30 min.; e) NaOMe, MeOH, rt, 3 h.

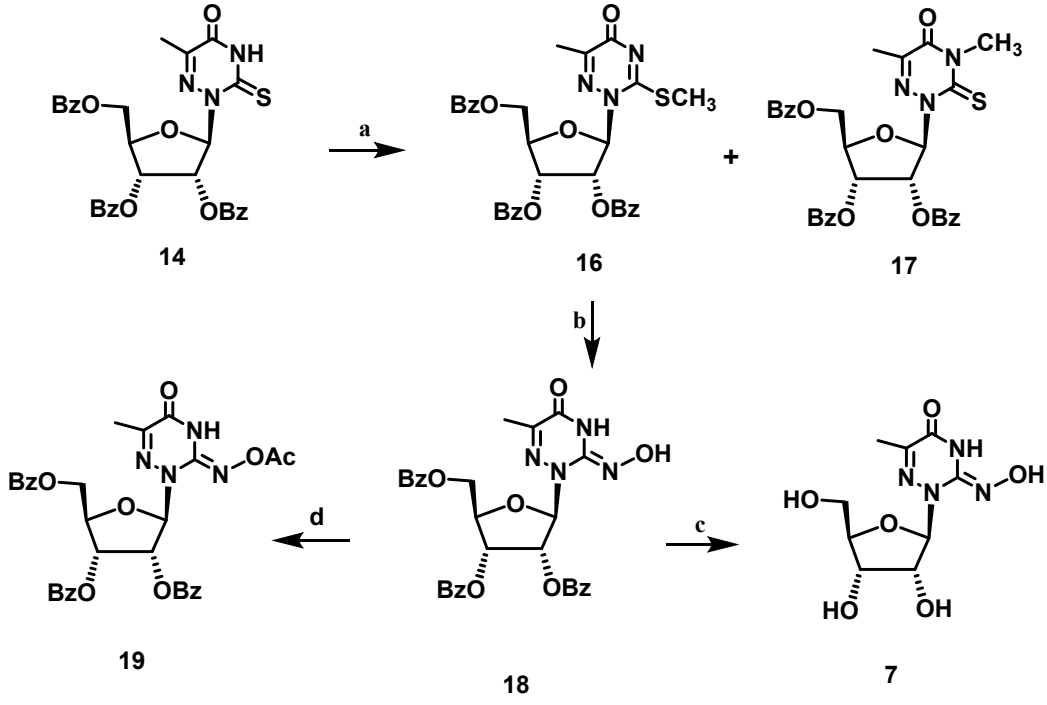
Table 1. Cyclization conditions of **11** to 6-aza-2-thiothymine (**12**).

Entry	Additive, No. equiv.	Solvent[a]	Time	Yield %
1	NaOAc, 8 / AcOH, 8		2.5 h	87[b]
2	NaOAc, 8 / AcOH, 8	EtOH	3 h	90[c]
3	NaOAc, 3 / AcOH, 3	EtOH	3.5 h	96[c]

All reactions were performed at 120 °C; b) Compound 12 was isolated as dark brown solid; c) Compound 12 was isolated as faint shiny yellow crystals.

Coupling of 6-aza-2-thiothymine (**12**) with the protected D-ribofuranosyl derivative **13** under Vorbrüggen conditions ($SnCl_4$/DCE) provided the 2-thio-6-aza-5-methyluracil derivative **14** in 89% yield. The N^1-β-configuration of the 2-thionucleoside derivative **14** was confirmed by ^{1}H, and ^{13}C-NMR. Treatment of **20** with TBSCl/DMAP/Et_3N, and then NH_3 in CH_3CN gave the corresponding cytidine derivative **21** in 73.5% yield. Deprotection of the hydroxyl groups of **14** and **15** with NaOMe/MeOH gave the corresponding free nucleosides **4**[41] and **5**, respectively in good yields (scheme 1). Treatment of **4** with H_2O_2 gave the corresponding 2-oxo derivative **6** [42] in good yield.

Scheme 2[a]



Reagents and conditions a) MeI, DBU, DMF, 0 °C-rt, 2 h; b) $NH_2OH{\cdot}HCl$, dry pyridine, 40 ºC, 48 h; c) NaOMe, MeOH, , rt, 3 h; d) Ac_2O, pyridine, 0 °C - rt, 3 h.

The synthesis of the 2-hydroxylimino derivative **7** is outlined in Scheme 2. Treatment of **14** with MeI in DMF in the presence of DBU gave 2-methylthio derivative **16** along with the corresponding N^3-methyl derivative **17** in 74.71% and 8.04% yields respectively. H-1' NMR signal of the 2-methylthio derivative **16** was shifted upfield at 6.32 ppm compared with that of the N^3-methyl derivative **17** at 7.33 ppm. Treatment of **16** with hydroxylamine hydrochloride in dry pyridine gave the corresponding 2-hydroxylimino derivative **18** in 83% yield. Treatment of **18** with Ac_2O/dry pyridine gave the corresponding mono-acetate derivative **19** in 78% yield. The protection of hydroxyl group in **19** as an acetate ester provides more lipophilicity as well as chemical stability at the C2 position of the nucleoside. Treatment of **18** with NaOMe/MeOH gave the corresponding free nucleoside **7** in 85% yield. Selective 1D-NOSEY (SELNOGP, supporting information) of **7** showed medium correlation between N^3-H and C^2-hydoxylimino moieties, and between 5'-OH and C^2=NOH moieties, supporting the *E*-configuration around the C^2=NOH side chain (Fig. 3).

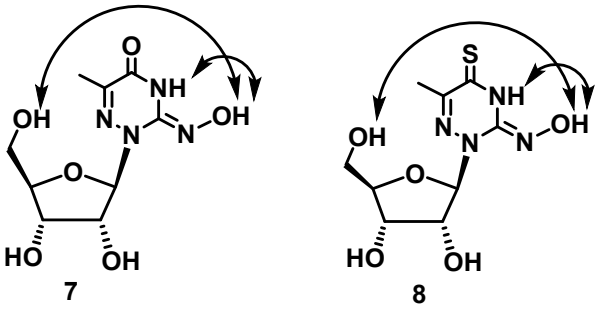
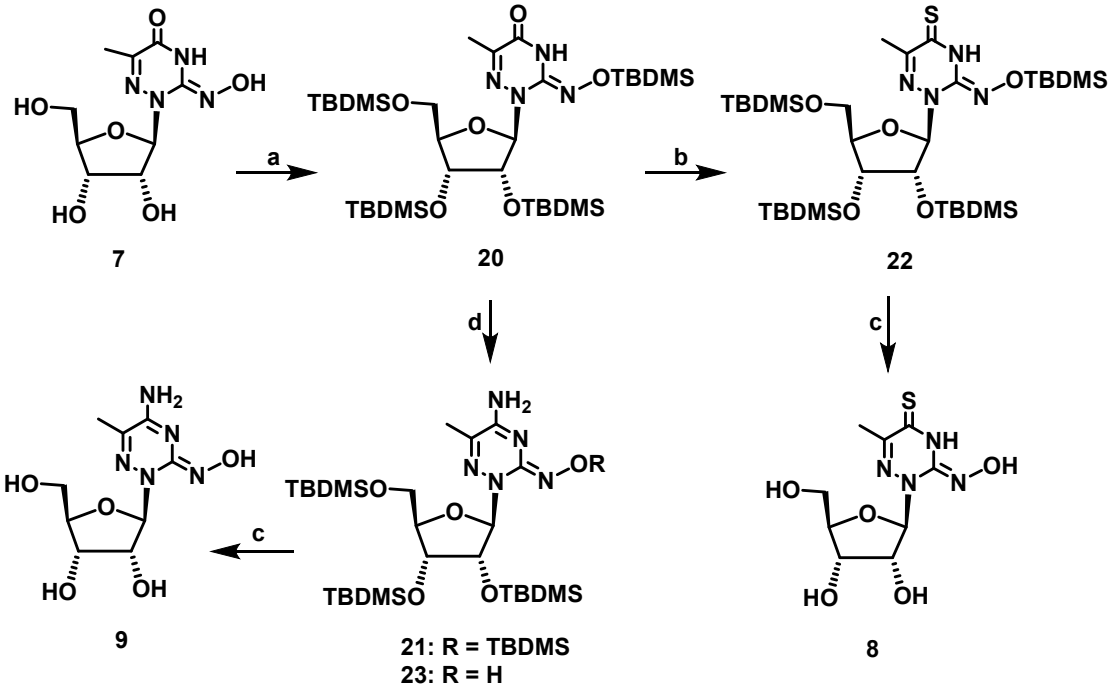


Fig. 3. Selective 1D-NOESY of compounds 7 and 8 in (DMSO-d_6). The observed NOE correlation between N^3-H and C^2=NOH, and the later with 5'-OH shown with arrows.

The synthesis of 4-thiono and 4-amino derivatives of **8** and **9** is shown in scheme 3. First, **7** was treated with TBDMSCl/Im/DMF to give the fully protected nucleoside **20** in 87.4% yield. Compound **20** in turn was treated with Lawesson's reagent to give the 4-thiono derivative **22** in 65.3% yield, which was then treated with TBAF/THF to give **8**. To make target **9**, compound **20** was treated with TPSCl/DMAP/Et_3N, and then with NH_3 gas to give the 4-amino derivative **21**; and the latter was finally deprotected by TBAF. Although the TBDMS group at the C^2-hydroxyimino of **20** underwent partial aminolysis to give the oxime derivative **23** as a side product, it is worth noting that no 2-amino derivative was formed due to the aminolysis.

Scheme 3[a]



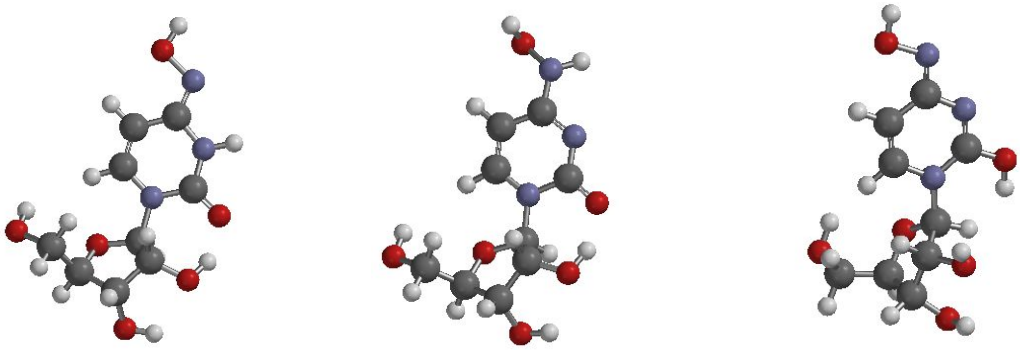
[a]Reagents and conditions. a) TBDMSCl, Im., DMF, , rt, 24 h; b) Lawesson's reagent, toluene, reflux, overnight; c)TBAF, AcOH, THF, , rt, 3 h; d) TBSCl, DMAP, CH_3CN., TEA, rt, 12 h, then NH_3 gas, 0 ºC 30 min.

Selective 1D-NOSEY (SELNOGP, supporting information) of **8** showed a medium correlation between the N^3-H and C^2-hydoxylimino moieties, and between the 5'-OH and the C^2=NOH moieties, supporting the *E*-configuration around the C^2=NOH structure (Fig. 3).

DFT calculations

It has been reported that NHC-triphosphate derivative is incorporated into HRSV's viral RNA in place of uridine, implying that NHC's nucleobase has a dual base pairing character (cytosine and uracil) as a result of placing a hydroxylamine moiety at the C^4-position of the nucleoside. To investigate whether placing the NHOH moiety at the C^2-position could results in a nucleoside with dual base pairing (uridine and isocytidine) characteristics, the tautomeric distributions of **7**, **8**, and **9** were calculated, in parallel with those of NHC, using DFT implemented in Spartan 18, at B3LYP/6-311+G** basis set [43-45]. Tautomer distribution results for NHC in aqueous environment

showed close Boltzmann weights for the uridine like tautomer (NHC-I) and cytidine like tautomer (NHC-II, 0.373) (Fig. 4) (see supporting information).



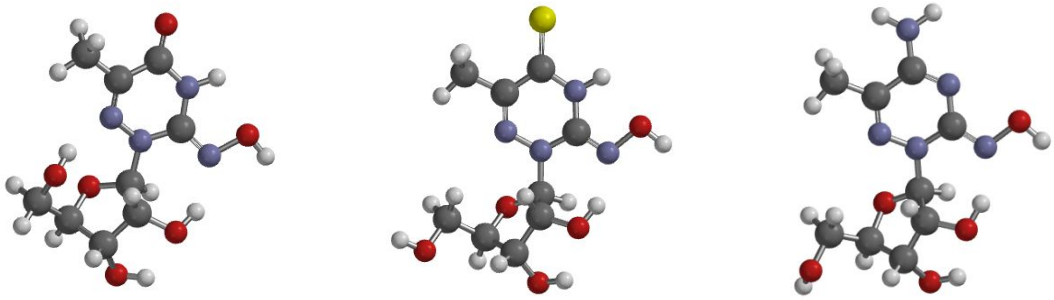
NHC-I Boltzmann Weight= 0.627

NHC-II Boltzmann Weight = 0.373

NHC-III Boltzmann Weight = 0.000

Fig. 4. Optimized structures of NHC tautomers at B3LYP/6-311+G** basis set

The tautomeric distribution for both geometrical isomers; (*E*) and (*Z*)-C^2=NOH of **7**, **8**, and **9** were calculated under the same conditions used with NHC (supporting information). A predominance of the (*E*)-C^2=NOH tautomer was observed with Boltzmann weight for **7** (EO-I; 0.997), **8** (ES-I, 0.986), and **9** (EA-I, 1.000) (Fig. 5). These results come in accordance with the observed NOE data (Fig. 5).

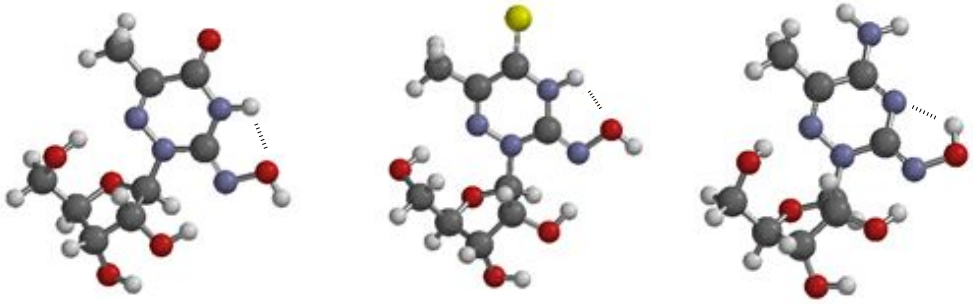


7 (EO-I) **8** (ES-I) **9** (EA-I)

Fig. 5. Optimized tautomeric structures of **7, 8,** and **9** at B3LYP/6-311+G** basis set.

Conformational distribution of the most stable tautomers for **7** (EO-I), **8** (ES-I), and **9** (EA-I) were assessed using a restricted hybrid HF-DFT SCF calculation at WB97X-D 6-31G* basis set (see supporting information). A preference of zigzag like conformation around the C^2=N-O-H bond

was shown for the most stable tautomers for **7** (EO-I-Conf. A), and **8** (ES-I-Conf. A), where the hydrogen atom of the C^2=N-O-H moiety is pointed away from the ring N^3-H, allowing hydrogen bonding between the oxygen's lone pair of electrons of the C^2=N-O-H moiety and the N^3-H (Fig. 6). A conformational preference around the N-O-H of the C^2=N-O-H moiety of **9** (EA-I-Conf. A) enables hydrogen bonding between the N^3 lone pair of electrons and the hydrogen atom of C^2=N-O-H moiety, thereby forming a five membered ring (Fig. 6).



7: EO-I-Conf. A **8**: ES-I-Conf. A **9**: EA-I-Conf. A

Fig. 6. Optimized conformers of tautomers **7** (E-OI), **8** (ES-I), and **9** (EA-I) using DFT at WB97X-D 6-31G* basis set.

Overall, DFT calculations concluded that placing a hydroxylamine moiety at the C^4 position of a pyrimidine nucleoside, as in NHC, results in a dual nucleobase behavior, cytosine-1-yl and uracil-1-yl, thereby supporting the mutagenic characteristic of the nucleoside. On the other hand, placing the hydroxylamine moiety at the C^2 position of a pyrimidine nucleoside supports its existence predominantly in the (*E*)-C^2=NOH form forming a purine like nucleoside through hydrogen bonding between the (*E*)-C^2=N-O-H moiety and the N^3-H or N^3-lone pair of electrons.

Antiviral activity

The novel free nucleoside analogs **4-9** and their intermediates **11**, **12**, **14**, and **16-19** were screened against several single stranded RNA viruses at Institute for Antiviral Research, Utah State University. The compounds were tested for their antiviral activity at four different concentrations by visual and neutral red (cytopathic effect/toxicity) assays. Nucleoside analogs that showed antiviral activity in the primary screening were further tested in dose response experiments at 8 different concentrations to determine the EC_{50}. Cytotoxicity (CC_{50}) was determined in parallel in uninfected cells.

Compounds were tested against the following viral strains: MR766 (Zika Virus) in Vero76 cell line; CIN-1, genotype 1 (hepatitis C) in Huh-7 cell line; EMC (MERS coronavirus) in Vero76 cell line; A2 (respiratory syncytial virus) in MA-104 cell line; California/07/2009 (Influenza A virus H1N1) in MDCK cell line; Kern 5156, WNo2 (west Nile Virus) in Vero76 cell line; 17D (Yellow fever Virus) in Vero 76 cell line. DMSO was used as vehicle in testing the in vitro antiviral activity of the tested compounds. Screening results for **4, 5, 7-9** and the intermediates **11**, **12**, **14**, **16-19** against respiratory viruses; MERS-CoV, HRSV and Flu A are shown in Table 2. Among the tested compounds, 4-thiono-2-hydroxylimino derivative **8** showed a significant activity against HRSV (EC_{50} = 6.1 μM, CC_{50} > 100 μM, SI >18) and was inactive against the other two respiratory viruses (Table 2). The observed activity of **8** against HRSV could be attributed to its potential mutagenic activity as a result of its existence in two forms, uridine-like (ES-I) and hydroxyisocytidine-like (ES-II) tautomers (Fig. 1, supporting information). On the other hand, the corresponding 4-oxo-2-hydroxylimino derivative **7** was lacking activity and cytotoxicity at the highest tested concentration.

Table 2. Anti-respiratory viral activity of nucleosides 5, 6, 8-12, 17-19.

Compd.	MERS-CoV[d]			HRSV[d]			Flu -A[d] (H1N1)[e]		
	[a]EC_{50} μM	[b]CC_{50} μM	[c]SI_{50}	EC_{50} μM	CC_{50} μM	SI_{50}	EC_{50} μM	CC_{50} μM	SI_{50}
11	>100	>100		>100	>100		>100	>100	
12	>100	>100		>100	>100		>100	>100	
4	>100	>100		>100	>100		>100	>100	
5	>100	>100		>100	>100		>100	>100	
7	>100	>100		>100	>100		>100	>100	
8	>100	>100		6.1	>100	>16	>100	>100	
9	>100	>100		>100	>100		>100	>100	
14	>4.2	4.2		>4.2	4.2		>6.6	6.6	
16	>56	56		>48	48		>100	>100	
17	>100	>100		>100	>100		>100	>100	
18	>32	32		32	32		>34	34	
19	>24	24		5.2	33	6	>12	12	

[a]EC_{50}: effective concentration that inhibit the replication of the virus by 50%. [b]CC_{50}: cytotoxic concentration that inhibit the replication of normal cells by 50%. [c]SI = CC_{50}/EC_{50}. [d]EC_{50} > 100 indicates no antiviral activity was observed at the tested highest concentration. [e] California/07/2009 isolate. Ribavirin was used as a control for HRSV.

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The carbazone derivative **11**, its cyclized form **12**, and the 2-thio-nucleosides **4**, **5** were devoid of antirespiratory viral activity and cytotoxicity at the highest tested concentration. Interestingly, the

2',3',5'-tri-*O*-benzoyl-2-hydroxylimino derivative **18** showed higher activity against HRSV (EC_{50} = 23 μM), compared with the free nucleoside **7** (EC_{50} = >100 μM), and the activity was further increased with the acetyl protected 2-hydroxylimino derivative **19** (EC_{50} = 5.3 μM, CC_{50} = 33 μM, SI = 6). The benzoyl protected 2-thio-6-aza-5-methyluridine derivative **14** showed activity against MERS-CoV, HRSV, and Flu-A viruses at the same cytotoxic concentration (Table 2). The activity/toxicity of the latter compound was lashed out upon methylation at the N-3 position, as in compound **17**. On the other hand, the activity/toxicity reappeared upon methylation at the 2-position of **14** as in **16** (Table 2), suggesting that **14** might act as a nucleoside analogue, once it gets into cells in high concentration. The lipophilic characteristics of the benzoyl groups in **14**, **16**, **18**, **19**, and the acetyl group in the latter compound could enhance the uptake of the compounds, and consequently their biological activity. Whether those protected nucleosides act as enzyme allosteric inhibitor(s) or as nucleoside analogues, that are activated to the corresponding nucleoside triphosphates is under investigation and will be reported in due course.

Compounds **4-9** and **11**, **12**, **14**, **16-19** were also screened against selected members of flaviviruses: ZIKA, WNV, YFV, HCV, and DENV-2 (Table 3). It is notable that the benzoyl protected nucleosides **14**, **18**, and **19** were also active against ZIKA, WNV, and YFV with SI_{50} in the range of 8.8-7.2 (Table 3).

Compd.	ZIKA[d]			WNV[d]			YFV[d]			HCV		
	[a]EC_{50} μM	[b]CC_{50} μM	[c]SI_{50}	EC_{50} μM	CC_{50} μM	SI_{50}	EC_{50} μM	CC_{50} μM	SI_{50}	EC_{50} μM	CC_{50} μM	SI_{50}
11	>100	>100		>100	>100		>100	>100		ND		
12	>100	>100		>100	>100		>100	>100		ND		
4	ND			ND			ND			>20	>20	1
5	>100	>100		>100	>100		>100	>100		ND		
6	>100	>100		>100	>100		>100	>100		ND		
7	>100	>100		>100	>100		>100	>100		7.74	>20	3
8	2.4	>100	>24	>100	>100		>100	>100		ND		
9	>100	>100		>100	>100		>100	>100		ND		
14	3.2	24	7.2	>3.2	3.2		>3.2	3.2		ND		
16	>100	>100		>28	28		>56	56		ND		
17	>100	>100		>42	42		>52	52		ND		
18	3.2	28	8.8	>3.2	3.2		>3.2	3.2		ND		
19	3.2	28	8.8	>3.2	3.2		>2.4	2.4		ND		

[a]EC_{50}: effective concentration that inhibit the replication of the virus by 50%. [b]CC_{50}: cytotoxic concentration that inhibit the replication of normal cells by 50%. [c]SI = CC_{50}/EC_{50}. [d]EC_{50} > 100

indicates no antiviral activity was observed at the tested highest concentration. 6-Azauridine was used as a control for Zika virus.

Table 3. Activity of nucleosides 4, 5, 7-9, 14, 16-19 against some *flaviviruses*.

Interestingly, the 4-thiono-2-hydroxylimino derivative **8** showed selective activity against ZIKA (EC_{50} = 2.4 μM, CC_{50} = >100 μM). The 2-hydroxylimino-6-aza-5-methyluridine (**7**) showed a moderate activity against HCV (EC_{50} = 7.74 μM) while it was inactive against the rest of the tested flaviviruses (Table 3). The cytidine analogue **5**, **9** were inactive against all tested viruses at the highest tested concentration.

Conclusions

We have synthesized for the first time a series of 6-azauridine analogues probing the replacement of the oxygen atom at the 2-position with hydroxylimin moiety on the anti-RNA viral activity. DFT results support a dual base pairing character for NHC while supporting a purine-like nucleobase for 2-hydroxyimino nucleosides. A significant activity against HRSV (EC_{50} 6.1 μM and SI >16) and ZIKA (EC_{50} 2.4 μM, CC_{50} >100 μM) were observed with 2-hydroxylimino-4-thionouridine derivative **8**. Unexpectedly, the fully benzoyl protected 2-hydroxylimino-6-azauridine derivative **19** showed significant activity against HRSV (EC_{50}= 5.2 μM) and ZIKA (EC_{50} 24 μM). Moderate activity against HCV (EC_{50} = 7.1 μM) was observed with 2-hydroxyliminouridine derivative **7**. The mechanisms of antiviral activity of the sugar protected nucleosides **14-16** and **18-19** is under investigation and will be reported in due course. This study shows that the 2-hydroxylimino-6-aza-5-methyluracil derived nucleosides provide lead compounds for further development of broad spectrum antiviral agents.

Acknowledgments.

Applied Nucleic Acids Research Center, Zagazig University utilized the non-clinical and pre-clinical services program offered by the National Institute of Allergy and Infectious Diseases. This work is supported by STDF Grants No. 2698/ 4603/5904 and by USA National Science Foundaiton CHE-1845486.

Protocols and experimental results.

Material, instruments and general considerations. Starting materials and reagents were purchased from Acros-Organics, Alpha Aeser, and Sigma-Aldrich. Reaction progress was monitored by TLC analysis using aluminum-backed plates pre-coated with Merck silica gel 60-F254. Column chromatography was carried out on Agela Technologies Flash silica 40-60 mesh. Melting points recorded on Electrothermal IA 9100 apparatus and were uncorrected. IR spectra (ATR) were recorded on Bruker alpha spectrometer. UV-vis spectra were recorded on Agilent Cary 60. ^{1}H and ^{13}C-NMR spectra were recorded on Bruker 400 MHz spectrometer. Elemental analysis were recorded on Vario MICRO Cube elemental analyzer. Computational details: all the structures of the compounds were built using Wavefunction Spartan 18 V2.0.7 and subjected to energy minimization to remove their strain energies. DFT was adopted in calculating the equilibrium geometry and conformational distribution using B3LYP/6-311+G** and WB97X-D 6-31G* basis sets, respectively.

Antiviral protocols.

Primary CPE test method. Four-concentration CPE inhibition assays are performed on confluent or near-confluent cell culture monolayers in 96-well plates. Cells are maintained in MEM or DMEM supplemented with FBS as required for each cell line. For antiviral assays the same medium is used but with FBS reduced to 2% or less and supplemented with 50 μg/ml gentamicin (10 μg/mL EDTA and 1 IU/mL trypsin are added for influenza virus). The test compound is prepared at four final concentrations, usually 0.1, 1.0, 10, and 100 μg/ml or μM. Five microwells are used per dilution: three for infected cultures and two for uninfected toxicity evaluation run in parallel. Controls for the experiment consist of six microwells that are infected but not treated (virus controls) and six that are untreated (cell controls). The virus control and cell control wells are on every microplate. Growth medium is removed from the 96-well plates of cells, then the test compound is applied in 0.1 ml volume to wells at 2x concentration. Virus, normally <100 $CCID_{50}$ (50% cell culture infectious doses) in 0.1 ml volume, is added to wells designated for virus infection. Medium devoid of virus is placed in toxicity control wells and cell control wells. Plates are incubated at 37°C (33°C for rhinovirus and enterovirus-D68) with 5% CO_2 until maximum CPE is observed microscopically in virus control wells. Plates are then stained with 0.011% neutral red for approximately two hours at 37°C with 5% CO_2. The neutral red medium is removed by complete aspiration, and the cells rinsed with phosphate buffered saline (PBS) to remove residual

dye. The PBS is completely removed and the incorporated neutral red is eluted with 50% Sorensen's citrate buffer/50% ethanol for at least 30 minutes. Neutral red dye penetrates into living cells, thus, the more intense the red color, the larger the number of viable cells present. The dye content in each well is quantified by optical density (OD) on a spectrophotometer at 540 nm wavelength. The OD for each set of wells is converted to a percentage compared to untreated control wells using a Microsoft Excel™ computer-based spreadsheet. Infected wells are normalized to the virus control. The 50% effective (EC_{50}, virus-inhibitory) concentrations and 50% cytotoxic (CC_{50}, cell-inhibitory) concentrations are then calculated by regression analysis. The quotient of CC_{50} divided by EC_{50} gives the selectivity index (SI_{50}) value. The percent CPE in each well may also be read microscopically, calculated as above, and reported as a second data set from the same plate for verification [46-48].

Secondary assay method: CPE and virus yield reduction (VYR). The secondary assay confirms the CPE assay result, with the principal assessment being virus yield reduction (VYR). It employs a similar method as described for the primary assay in section 2D.8.1, with the differences noted in this section. The secondary assay is run independently of the primary test with cells, culture media, infectious virus solution, and test compound dilutions all newly prepared for this assay. Eight half-log_{10} concentrations are tested for antiviral activity and cytotoxicity. After sufficient virus replication occurs (normally 3 days post infection), a sample of supernatant is taken from each infected well (three replicate wells are pooled) and titrated immediately (as with HRSV) or frozen and stored for virus titration at a later time. Alternately, a separate plate may be prepared and the plate may be frozen and then thawed to release intracellular or cell-associated virus to test the cell lysate rather than supernatant fluid. After maximum CPE is observed, the plates are stained with neutral red dye as described above to generate the neutral red EC_{50}, CC_{50}, and SI_{50} values. Uninfected wells are tested in parallel for compound toxicity as in the primary assay explained above. The positive control compound is evaluated in parallel with each test. The second step is to determine the virus titer in the supernatant or lysate samples. Samples from triplicate wells, collected as described above, are pooled and tested by endpoint dilution [49]. This is a direct determination virus produced in the presence of the test compound compared to virus from untreated, infected controls. This is accomplished by making log_{10} dilutions of the pooled samples and plating each dilution on 3 or 4 microwells with fresh monolayers of cells in 96-well plates. Plates are incubated until maximum CPE is observed, then each well is scored for presence (+) or

absence (-) of virus and the virus titer calculated using the Reed-Meunch method [50]. Plotting the log of the inhibitor concentration versus $\log_{10}$ of virus produced at each concentration allows calculation of the 90% (one $\log_{10}$ reduction) effective concentration by linear regression. Dividing EC_{90} by the CC_{50} toxicity obtained in the CPE assay gives the SI_{90} value (so named because it is derived from a 90% virus-inhibitory value).

Chemistry

(*Z*)-Ethyl 2-(2-carbamothioylhydrazono)propanoate (11). To a solution of **10** (10 g, 0.11 mol) in absolute ethanol (30 mL), was added ethyl pyruvate (15.3 mL). Then the solution was stirred for 2 hours at reflux temperature. The reaction mixture was cooled to room temperature and the precipitate was filtered off, dried under vacuum to give compound **11**[51] (19.08 g, 95.4% yield) as a white solid. M. p.,149-151 ºC; UV-vis (MeOH) λ_{max} 300 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm:10.66 (s,1H, NH), 8.66 (s, 1H, NH), 7.64 (s, 1H, NH) 4.18 (q, J = 6.8 Hz, 2H, C$\underline{H_2}$CH$_3$), 2.09 (s, 3H, CH$_3$), 1.24 (t, J = 6.8 Hz, 3H, CH$_2$C$\underline{H_3}$); ^{13}C APT NMR (100 MHz, DMSO-d_6) δ ppm: 179.92 (C=S), 164.20 (C=O), 139.02 (C=N), 61.11 ($\underline{C}$H$_2$CH$_3$) 14.00 (CH$_3$), 13.04 ($\underline{C}$H$_3$); IR (ATR, cm^{-1}): 3434.71 (νN-H), 3175 (νNH$_2$), 1702 (νC=O), 1594.44 (νC=S); Anal cald For $C_6H_{11}N_3O_2S$: C, 38.08; H, 5.86; N, 22.21. Found: C, 37.98; H, 5.74; N, 22.08.

6-Aza-5-methyl-2-thiouracil (12). A solution of compound **11** (16.51 g, 0.087 mol) and anhydrous sodium acetate (57.27 g, 0.698 mol) in (1/1 *v/v* glacial acetic acid/absolute ethanol, 80 mL) was heated for 2.5 hours at 110 ºC. The reaction was cooled to room temperature, quenched with cold saturated sodium bicarbonate solution, and then extracted with ethyl acetate. The organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum. The residue was purified by silica gel column chromatography (eluate: ethyl acetate) to give compound **12**[51] (11.99 g, 96% yield) as a yellow crystal: M. p., 222-224 ºC; UV-vis (MeOH, nm) λ_{max} 269.98, 214.98 (shoulder); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 13.25 (s, 1H, NH), 13.00 (s, 1H, NH), 2.06 (s, 3H, CH$_3$); ^{13}C APT NMR (100 MHz, DMSO-d_6) δ ppm: 173.36 (C=S), 153.43 (C=O), 148.42 (C=N), 16.03 (CH$_3$). IR (ATR, cm^{-1}): 3041.60 (νN-H), 2892.47 (νN-H), 1676.04 (νC=O) 1599.58 (νC=S); Anal cald For $C_4H_5N_3OS$: C, 33.56; H, 3.52; N, 29.35. Found: C,33.66 ; H, 3.58; N, 29.18.

1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (13)[39]. Sulfuric acid (0.5 mL) in methanol (1 mL) was added dropwise to a suspension of D(-)ribose (5 g; 33.3 mmol) in methanol (100 mL). The mixture was stirred for 5 hours at room temperature, and then the reaction was quenched with sodium carbonate (10 g). The reaction mixture was filtered and the filtrate was concentrated under reduced pressure to afford 7.79 g of crude 1-*O*-methyl-D-ribofuranose as a yellowish syrup. The crude 1-*O*-methyl-D-ribofuranose (5.46 g) was dissolved dry pyridine (16 mL) and the solution was cooled to 0 ºC and benzoyl chloride (15.56 mL) was added dropwise, the reaction mixture was stirred for 12 hours at room temperature. The volatiles were evaporated and co-evaporated with toluene. The residue was diluted with ethyl acetate, washed with saturated solution of $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give of 2,3,5-tri-*O*-benzoyl-1-*O*-methyl-D-ribofuranose (11 g) as a colorless syrup. To a solution of 7.55 g of the crude 2,3,5-tri-*O*-benzoyl-1-*O*-methyl-D-ribofuranose in acetic acid (13.2 mL) and acetic anhydride (2.25 mL), conc. H_2SO_4 (0.85 mL) was added dropwise at 0 ºC. The reaction mixture was stirred for 5 hours at room temperature, and then reaction was quenched with saturated solution $NaHCO_3$. The whole was partitioned between with ethyl acetate and water. The organic layer was separated, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was crystallized from *iso*-propanol to give a white crystal: ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.90-8.07 (m, 2H, Ar), 8.02-7.99 (m, 2H, Ar), 7.90-7.88 (m, 2H, Ar),7.61-7.51 (m, 3H, Ar), 7.45-7.32 (m, 6H, Ar), 6.43 (s, 1H, H-1), 5.92-5.89 (m, 1H, H-2), 5.79-7.78 (m, 1H, H-3), 4.81-7.75 (m, 2H, H-4 and H-5), 4.54-4.49 (m, 1H, H-5), 2.00 (s, 1H, $C\underline{H}_3$); APT ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 169.23 (C=O), 166.14 (C=O), 165.52 (C=O),165.18 (C=O), 133.83 (Ar),133.72 (Ar), 133.42 (Ar), 130.01 (Ar), 129.92 (Ar), 129.74 (Ar), 128.98 (Ar), 128.82 (Ar), 128.70 (Ar), 128.57 (Ar), 128.56 (Ar), 98.56 (C-1), 80.13 (C-2), 75.15 (C-3),71.53 (C-4), 63.88 (C-5), 21.05 ($\underline{C}H_3$); IR (ATR, cm^{-1}): 1720 (νC=O).

1-(2',3',5'-tri-*O*-benzoyl-β-D-ribofuranosyl)-6-aza-5-methyl-2-thiouracil (14). To a suspension of **12** (2 g, 13.9 mmol) and **13** (7.39 g 14 mmol) in dry DCE (200 mL) was added BSA (4.15 mL, 16.7 mmol) dropwise at room temperature. The solution was stirred for 30 minutes until complete dissolution. Then $SnCl_4$ (4.91 mL, 16.7 mmol) was added dropwise at -10 ºC. The reaction mixture was stirred 3 hours at 0 ºC, then the reaction was quenched by DMSO (5.9 mL),

and the formed solid was filtered off. The filtrate was neutralized with saturated aqueous $NaHCO_3$ solution. The whole was extracted with DCM and the combined organic phases were dried over anhydrous Na_2SO_4, and evaporated under vacuum. The residue was purified with silica gel column chromatography (eluate: 2% ethyl acetate / DCM),to give compound **14** (7.298 g, 89% yield) as a white solid: M. p., 159-161 ºC; UV-vis (MeOH) λ_{max} 275 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 13.35 (s, 1H, NH), 7.98-7.39(m, 15H, Bz), 7.35 (d, J = 2.8Hz, 1H, H-1'), 5.98 (dd, J = 2.8, J = 5.6 Hz, 1H, H-2'), 5.92 (dd, J = 5.6, J = 6.8 Hz, 1H H-3'), 4.83 (m, 1H, H-4'), 4.70 (dd, J = 4.7, 12.4 Hz, 1H, H-5'a), 4.58 (dd, ,J = 4.8, 12.4 Hz, 1H, H-5'$_b$), 2.02 (s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO - d_6) δ ppm:174.55 (C=S), 165.34 (C=O), 164.56 (C=O), 164.50 (C=O), 152.11 (C=O), 150.03 (C=N), 134 (Ar), 133.82 (Ar), 133.55 (Ar), 129.41 (Ar), 129.21 (Ar), 128.87 (Ar), 128.74 (Ar), 128.70 (Ar), 128.51 (Ar), 128.39 (Ar), 91.10 (C-1'), 78.98 (C-2'), 74.11 (C-3'), 70.96(C-4'), 63.31 (C-5'), 16.25 (CH_3); IR (ATR, cm^{-1}): 3215.85 (νN-H), 1735.65 (νC=O), 1707.34 (νC=N), 1600.94 (νC=S); EI-Mass m/z 587 [M$^+$]; Anal cald For $C_{30}H_{25}N_3O_8S$: C, 61.32; H, 4.29; N, 7.15. Found: C, 61.49; H, 4.36; N, 6.98.

1-(2',3',5'-Tri-*O*-benzoyl-β-D-ribofuranosyl)6-aza-5-methyl-2-thiocytosine (15). A mixture compound **14** (0.5 g, 0.85 mmol), TBSCl (0.64 g, 2.128 mmol) and DMAP (0.259 g, 2.13 mmol) were dissolved in dry acetonitrile (10 mL), then TEA (0.295 mL) was added dropwise at 0 ºC. The mixture was stirred for 12 hours at room temperature, then ammonia gas was bubbled for 30 min. at ºC and the mixture was stirred for 30 minutes at room temperature. The solvents were evaporated under vacuum and the residue was purified by silica gel column chromatography (eluate: 5% methanol / DCM) to give compound **15** (0.374 g, 75% yield) as a pale yellow crystals: M. p., 106-111 ºC; UV-vis (MeOH) λ_{max} 275, 230 nm;^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 7.95-7.90 (m, 6H, Ar), 7.67-7.65 (m, 3H, Ar), 7.51-7.44 (m, 8H, Ar and 4-NH_2, 2H were exchangeable with D_2O), 6.35 (br s , 1H, H-1'), 6.07 (br d, 1H, H-2'), 5.92 (dd, J = 5.6, J = 6.4 Hz, 1H, H-3'), 4.78 (br d, 1H, H-4'), 4.69 (br dd, J = 12.4 Hz, 1H, H-5a'), 4.58 (br dd, J = 12.4 Hz, 1H, H-5b'), 1.91 (s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 165.32 (C=S), 164.68 (C4), 164.52 (C=O), 162.11 (C=O), 155.48 (C=N), 148.11 (C=N), 133.97 (Ar), 133.83 (Ar), 133.54 (Ar), 129.34 (Ar), 129.32 (Ar), 129.21 (Ar), 129.13 (Ar), 128.82 (Ar), 128.74 (Ar), 128.69 (Ar), 128.57 (Ar), 128.53 (Ar), 89.49 (C-1'), 78.91 (C-2'), 73.70 (C-3'), 70.76 (C-4'), 62.92 (C-5'), 16.80 (CH_3); IR (ATR, cm^{-1}): 3327.39 (νNH$_2$), 1721.95 (νC=O), 1621.92 (νC=N), 1515.81 (νC=S).

1-(β-D-Ribofuranosyl)6-aza-5-methyl-2-thiocytosine (5). A solution of compound **15** (0.35 g, 0.596 mmol) in dry methanol (5 mL) was treated with sodium methoxide (1*M*, 5.6 mL) at 0 ºC. The reaction mixture was stirred for 2 hours at room temperature, and then was neutralized with acetic acid. The solvents were removed under vacuum and the residue was purified by silica gel column chromatography (eluate: 5% methanol/DCM), then (8:1:1:1, EtOAc : EtOH : Acetone : H_2O) to give compound **5** (0.139 g, 85% yield) as a white solid: M. p., 237-238 ºC; UV-vis (MeOH) λ_{max} 245 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 7.32 (br s, 2H, NH_2, exchangeable with D_2O), 5.62 (d, J = 3.6 Hz, 1H, H-1'), 5.33 (br s, 1H, 2'-OH, exchangeable with D_2O), 5.06 (br s, 1H, 3'-OH, exchangeable with D_2O), 4.90 (br t, 1H, 5'-OH, exchangeable with D_2O), 4.37 (br s, 1H, H-2'), 4.04 (br s, 1H, H-3'), 3.85 (br d, 1H, H-4'), 3.56-3.40 (m, 2H, H-5'a and H5'b), 2.01 (s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 162.45 (C=S), 155.78 (C-4), 147.05 (C=N), 93.03 (C-1'), 85.02 (C-2'), 71.91 (C-3'), 70.29 (C-4'), 61.80 (C-5'), 16.89 (CH_3); IR (ATR, cm^{-1}): 3430.08 (νO-H), 3346.57 (ν OH), 3181.48 (ν OH), 2957.75-2933.87 (ν N-H_2), 1726.77 (νC4=N), 1668.49 (νC5=N), 1622.60 (νC=S); EI-Mass *m/z* 274.44 [M^+]; Anal cald For $C_9H_{14}N_4O_4S$: C, 39.41; H, 5.14; N, 20.43. Found: C, 39.37; H, 5.29; N, 20.22.

1-(β-D-Ribofuranosyl)6-aza-5-methyl-2-thiouracil (4). Sodium methoxide (1*M*, 7.99 mL) was added to a solution of compound **14** (0.5 g, 0.85 mmol) in dry methanol (5 mL) at 0 ºC. The reaction mixture was stirred for 3 hours at room temperature, neutralized with acetic acid, and evaporated under vacuum. The residue was purified by silica gel column chromatography (eluate: 10% methanol / DCM) to give compound **4** (0.1991 g, 85% yield) as a white solid granules: M. p., 220-224 ºC; (νC=S) ; UV-vis (MeOH) λ_{max} 275 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 13.19 (s, 1H, NH, exchangeable with D_2O), 6.79 (br d, 1H, H-1'), 5.28 (d, J = 4.8 Hz, 1H, 2'-OH, exchangeable with D_2O), 5.00 (d, J = 6.4 Hz, 1H, 3'-OH, exchangeable with D_2O), 4.59 (t, J = 5.6 Hz, 1H, 5'-OH, exchangeable with D_2O), 4.17 (m, 1H, H-2'), 4.06 (m, 1H, H-3'), 3.81 (m, 1H, H-4'), 3.57 (m, 1H, H-5'a), 3.44 (m, 1H, H-5'b), 2.1 (s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 174.44 (C=S), 152.18 (C=O), 149.08 (C=N), 93.67 (C-1'), 84.78 (C-2'), 73.4 (C-3'), 70.4 (C-4'), 62.04 (C-5'), 16.34 (CH_3); IR (ATR, cm^{-1}): 3472.02 (ν OH), 3426.22 (ν OH), 3178.14

(ν OH), 2886.77 (νN-H), 1694.17 (C=O), 1493.75; EI-Mass *m/z* 275.27 [M^+]. Anal cald For $C_9H_{13}N_3O_5S$: C, 39.27; H, 4.76; N, 15.26. Found: C, 39.41; H, 4.97; N, 15.41.

1-(β-D-Ribofuranosyl)-6-aza-5-methyluracil (6). A solution of compound **4** (50 mg, 0.1816 mmol) was dissolved in distilled water (2 mL) and treated with H_2O_2 (50% in H_2O, 74 μL, 1.0897 mmol), and NaOH (43.58 mg, 1.0897 mmol) at 0 ºC. The mixture was stirred for 1 hour at room temperature and the solvent was evaporated to dryness. The residue was purified by silica gel column chromatography (eluate: EtOAc) to give compound **6** (38 mg, 80.73% yield) as a white foam: UV-vis (MeOH) λ_{max} 210, 265 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 5.90 (d, J = 4 Hz, 1H, H-1'), 5.04 (d, J = 5.2 Hz, 1H, 2'-OH, exchangeable with D_2O), 4.84 (d, J = 5.6 Hz, 1H, 3'-OH, exchangeable with D_2O), 4.66 (t, 1H, J = 5.2 Hz, 5'-OH, exchangeable with D_2O), 4.20 (m, 1H, H-2'), 3.99 (m, 1H, H-3′), 3.72 (m, 1H, H-4'), 3.51 (dd, J = 4.4, 12 Hz, 1H, H-5'a), 3.38 (dd, J = 6, 11.6 Hz, 1H, H-5'b), 1.95 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 163.88 (C=O), 155.44 (C=O), 143.22 (C=N), 90.21 (C-1'), 84.56 (C-2'), 72.78 (C-3'), 71.20 (C-4'), 62.99 (C-5'), 17.43 (CH_3); IR (ATR) (cm^{-1}): 3255 cm^{-1} (ν OH), 1701 cm^{-1} (ν C=O), 1608cm^{-1}(νC=S), 1530 (νC=N); Anal cald For $C_9H_{13}N_3O_6$: C, 41.70; H, 5.06; N, 16.21. Found: C, 41.81; H, 5.13; N, 16.08.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribo-furanosyl)-6-aza-5-methyl-2-methylthiouracil (16) and 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-6-aza-*N*^{3}-methyl-5-methyl-2-thiouracil (17). To a solution of compound **14** (1.7 g, 2.89 mmol) in dry DMF (100 mL) and CH_3I (0.54 mL, 8.679 mmol), DBU (0.648 mL, 4.34 mmol) was added dropwise at 0 ºC and the reaction mixture was stirred for 2 hours at the same temperature. The mixture was diluted with ethyl acetate then washed with H_2O and brine. The combined organic phases were dried over anhydrous sodium sulfate, filtered off and evaporated under vacuum. The residue was purified by silica gel column chromatography (eluate: 4% ethyl acetate/DCM) to give compound **17** (0.139 g, 8.04% yield) as a white solid, then eluted with 10% ethyl acetate in DCM to give **16** (1.3 g, 74.71% yield) as a white glassy semi solid. Analytical data for **17**: M. p.,136-137 ºC; UV-vis (MeOH) λ_{max} 225 nm (shoulder), 275 nm; ^{1}H NMR(400MHz, $CDCl_3$) δ ppm: 8.04-7.92 (m, 6H, Ar), 7.58-7.52 (m, 4H, Ar), 7.42-7.33 (m, 6H, Ar and H-1'), 6.09 (dd, J = 2.8, 5.6 Hz, 1H, H-2'), 5.97 (br dd, 1H, H-3'),

4.81-4.75 (m, 2H, H-4'and H-5'a), 4.60 (m, 1H, H-5'b), 2.22 (s, 3H, 5-CH_3), 1.57 (s, 3H, N^3-CH_3); APT ^{13}C NMR (100 MHz, $CHCl_3$-d_6) δ ppm: 175.97 (C=S), 166.20 (C=O), 165.47 (C=O), 165.31 (C=O), 152.35 (C=O), 146.95 (C=N) 133.83 (Ar), 133.73 (Ar), 133.43 (Ar), 130.03 (Ar), 129.91 (Ar), 129.87 (Ar), 129.69 (Ar), 128.89 (Ar), 128.67 (Ar), 128.60 (Ar), 128.58 (Ar), 93.81 (C-1'), 79.98 (C-2'), 74.43 (C-3'), 71.61 (C-4'), , 63.64 (C-5'), 34.10 (N^3-CH_3), 17.29 (5-CH_3);IR (ATR, cm^{-1}): 1722.43 (νC=O), 1695.70 (νC=N) 1601.21 (νC=S); Anal cald For $C_{31}H_{27}N_3O_8S$: C, 61.89; H, 4.52; N, 6.98. Found: C, 61.84; H, 4.48; N, 6.89. Analytical data for Compound **16**: UV-vis (MeOH) λ_{max} 230 nm; ^{1}H NMR (400MHz, DMSO-d_6) δ ppm: 7.96-7.85 (m, 6H, Ar), 7.68-7.62 (m, 3H, Ar), 7.51-7.42 (m, 6H, Ar), 6.32 (d, J = 2.4 Hz, 1H, H-1'), 6.08 (dd, J = 2.4, 5.6 Hz, 1H, H-2'), 5.96 (dd, J = 5.6, 7.2 Hz, 1H, H-3'), 4.88 (m, 1H, H-4'), 4.70 (dd, J = 3.2, 12.4 Hz, 1H, H-5'a), 4.57 (dd, J = 4.0, 12.4 Hz, 1H, H-5'b), 2.5 (s, 3H, SCH_3), 2.1 (s, 3H, 5-CH_3); ^{13}C APT NMR (100 MHz, DMSO-d_6) δ ppm: 165.28 (C=O), 164.59 (C=O), 164.47 (C=O), 158.94 (C=O), 149.98 (C=N), 133.99 (Ar), 133.85 (Ar), 133.57 (Ar), 129.45 (Ar), 129.27 (Ar), 129.12 (Ar), 128.81 (Ar), 128.76 (Ar), 128.71 (Ar), 128.48 (Ar), 128.42 (Ar), 91.44 (C-1'), 79.52 (C-2'), 73.94 (C-3'), 70.55 (C-4'), , 62.67 (C-5'), 16.65 (5-CH_3), 13.84 (5-CH_3); IR (ATR, cm^{-1}): 1718.09 (νC=O), 1666.39 (νC=N), 1598.85 (νC=S); Anal cald For $C_{31}H_{27}N_3O_8S$: C, 61.89; H, 4.52; N, 6.98. Found: C, 61.92; H, 4.59; N, 6.84

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)6-aza-2-hydroxylimino-5-methyluracil (18). A mixture of compound **16** (1.3 g, 2.1 mmol) and hydroxylamine hydrochloride (3 g, 43.2 mmol) was dissolved in dry pyridine (25 mL). The reaction mixture was stirred for 48 hours at 40 ºC. Then pyridine was evaporated and co-evaporated with toluene. The residue was purified by silica gel column chromatography (eluate: ethyl acetate /DCM).to give compound **18** (1.051 g, 82.98% yield) as a green fluffy foam; UV-vis (MeOH) λ_{max} 330 nm (broad), 275 (shoulder), 230 nm; ^{1}H NMR (400MHz, DMSO-d_6) δ ppm: 11.01 (s , 1H, OH, exchangeable with D_2O), 9.58 (s, 1H, NH, exchangeable with D_2O), 8.00 -7.42 (m, 15H, Bz), 6.20 (d, J = 3.2Hz, 1H, H-1'), 6.02 (dd, J = 3.2, 5.2 Hz, 1H, H-2'), 5.86 (dd, J = 5.6, 6.0 Hz, 1H, H-3'), 4.72 (m, 1H, H-4'), 4.66 (dd, J = 3.6, 12 Hz, 1H, H-5'a), 4.54 (dd, J = 4.4, 12 Hz, 1H, H-5'b), 1.85 (s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 165.37 (C=O), 164.63 (C=O) , 164.58 (C=O), 153.70 (C=O), 143.41 (C=N), 138.77 (C=N), 133.95 (Ar), 133.81 (Ar), 133.55 (Ar), 129.36 (Ar) , 129.28 (Ar), 129.21 (Ar),

128.84 (Ar), 128.74 (Ar), 128.72 (Ar), 128.57 (Ar), 128.45 (Ar), 88.14 (C-1'), 78.36 (C-2'), 72.91 (C-3'), 71.00 (C-4'), 63.38 (C-5'), 15.94 (CH_3); IR (ATR, cm^{-1}): 3268.55 (νO-H), 3064.31(νNH oxime), 1717.26 (νC=O), 1657.59 (νC=N), 1600.74 (νC=N); EI-Mass *m/z* 587.13 [M^+]; Anal cald For $C_{30}H_{26}N_4O_9$: C, 61.43; H, 4.47; N, 9.55. Found: C, 61.55; H, 4.89; N, 9.32.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)6-aza-2-*O*-acetylhydroxyimino-5-methyluracil (19). Acetic anhydride (0.288 mL, 3.1 mmol) was added dropwise at 0 ºC to a solution of compound **18** (0.6 g, 1.02 mmol) in dry pyridine (15 mL). The reaction mixture was stirred for 3 hours at room temperature. The volatiles were evaporated and co-evaporated with toluene. The residue was purified by silica gel column chromatography (eluate: 10 %ethyl acetate /DCM).to give compound **19** (0.5 g, 77.7% yield) as a white foam: UV-vis (MeOH) λ_{max} 234, 274.98 (broad), 310 nm (broad); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 11.99 (s, 1H, N-H, exchangeable with D_2O), 7.99 -7.39 (m, 15H, Bz), 6.28 (d, *J* = 3.2 Hz, 1H, H-1'), 6.06 (dd, *J* = 3.2, 5.6 Hz, 1H, H-2'), 5.92 (br dd, 1H, H-3'), 4.77 (m, 1H, H-4'), 4.67 (dd, *J* = 3.2, 12.4 Hz, 1H, H-5'a), 4.56 (dd, *J* = 4.4, 12.4 Hz, 1H, H-5'b), 2.1 (s, 3H, $COCH_3$); 1.92(s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 167.48 (C=O), 165.36 (C=O), 164.61 (C=O), 164.56 (C=O), 153.73 (C=O), 148.88 (C=N),141.45(C=N) 133.97-133.55 (Ar), 129.43-128.42 (Ar), 89 (C-1'), 78.62 (C-2'), 73.13 (C-3'), 70.84 (C-4'), 63.35 (C-5'), 15.92 (CH_3), 14.08(CH_3); IR (ATR, cm^{-1}): 1769.9 (νC=O), 1717.41 (νC=O), 1631.9 (νC=N), 1600.07 (νC=N); Anal cald For $C_{32}H_{28}N_4O_{10}$: C, 61.14; H, 4.49; N, 8.91;. Found: C, 61.34; H, 4.51; N, 8.89.

1-(β-D-Ribofuranosyl)-6-aza-2-hydroxyimino-5-methyluracil (7). Sodium methoxide (1*M*, 64.1 mL) was added a solution of compound **18** (4 g, 6.82 mmol) in dry methanol (20 mL) at 0 ºC and the mixture was stirred for 3 hours at room temperature. The reaction mixture was neutralized with acetic acid, and then the solvents were evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluate: 8:1:1:1 ethyl acetate/acetone/ethanol/H_2O) to give compound **7** (1.588 g, 85% yield) as a green solid: M. p.,171-173ºC; UV-vis (MeOH) λ_{max} 330 , 230 nm (shoulder); ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 10.76 (s, 1H, NH, exchangeable with D_2O), 9.43 (s, 1H, N=OH, exchangeable with D_2O), 5.66 (d, *J* = 3.8 Hz, 1H, H-1'), 5.15 (d, *J* = 5.3 Hz, 1H, 2'-OH, exchangeable with D_2O), 4.93 (d, *J* = 5.8 Hz, 1H, 3'-OH, exchangeable with

D_2O), 4.63 (br t, 1H, 5'-OH, exchangeable with D_2O), 4.23 (m, 1H, H-2'), 3.98 (d, 1H, H-3'), 3.74 (m, 1H, H-4'), 3.49 (m, 1H, H-5'$_a$), 3.38 (m, 1H, H-5'$_b$), 1.98 (s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm:153.95 (C=O) 144.16 (C=N), 137.03 (C=N), 90.26 (C-1'), 84.25 (C-2'), 71.92 (C-3'), 70.37 (C-4'), 62.21 (C-5'), 16.08 (CH_3); IR (ATR, cm^{-1}): 3374.72 (νN-H), 3262.42 (ν OH), 1691.18 (νC=O), 1652.82 (νC=N); EI-Mass *m/z* 274.18 [M$^+$]; Anal cald For $C_9H_{14}N_4O_6$: C, 39.42; H, 5.15; N, 20.43; found: C, 39.13; H, 5.27; N, 20.33.

2,2',3',5'-Tetra-*O-tert*-butyldimethylsilyl-β-D-ribofuranosyl-6-aza-2-hydoxyimino-5-methyluridine (20). A mixture of compound **7** (1.5 g, 5.47 mmol), TBDMSCl (11.543 g, 76.59 mmol) and imidazole (10.428 g, 153.1 mol) were dissolved in dry DMF (10 mL) and stirred for 24 hours at room temperature. The mixture was quenched with ice-H_2O, diluted with ethyl acetate and then washed with water. The organic phases were dried over anhydrous sodium sulfate, filtered off, and evaporated under vacuum. The residue was purified by silica gel column chromatography (eluate: 5% ethyl acetate /hexane) to give compound **20** (3.497 g, 87.4% yield) as a greenish syrup: UV-vis (MeOH) λ_{max} 335, 235 nm (shoulder); ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.74 (s, 1H, NH), 5.88 (d, *J* = 4.8 Hz, 1H, H-1'), 4.42 (dd, *J* = 4.4 Hz, 1H, H-2'), 4.28 (dd, *J* = 4.0 Hz, 1H, H-3'), 3.93 (m, 1H, H-4'), 3.72-3.63 (m, 2H, H-5'$_{a,b}$), 2.13 (s, 3H, 5-CH_3), 0.89 (m, 36H, *t*-Bu-Si), 0.1(m, 24H, CH_3-Si); APT ^{13}C NMR (100 MHz, $CDCl_3$-d_6) δ ppm: 153.58 (C=O), 146.85 (C=N), 137.76 (C=N), 89.04 (C-1'), 84.16 (C-2'), 73.49 (C-3'), 71.90 (C-4'), 62.84 (C-5'), 26.36 (t-Bu-Si), 26.06 (t-Bu-Si), 25.99 (t-Bu-Si), 25.88 (t-Bu-Si), 25.79 (t-Bu-Si), 18.56 (t-Bu-Si), 18.45 (t-Bu-Si), 18.21 (t-Bu-Si),16.29 (5-CH_3), 0.14 (CH_3-Si), -3.43 (CH_3Si), -4.25 (CH_3-Si), -4.37 (CH_3-Si), -4.43 (CH_3-Si), -4.58 (CH_3-Si), -5.18 (CH_3-Si), -5.21(CH_3-Si), -5.25 (CH_3-Si). IR (ATR, cm^{-1}): 2952.77 cm^{-1} (νN-H), 1697.88 (νC=O), 1649.62 (νC=N).

2,2',3',5'-Tetra-*O-tert*-butyldimethylsilyl-β-D-ribo-furanosyl-6-aza-2-hydoxyimino-5-methylcytidine (21) and 2',3',5'-tri-*O-tert*-butyldimethylsilyl-β-D-ribo-furanosyl-6-aza-2-hydoxyimino-5-methylcytidine (23). A mixture of compound **20** (1.5 g, 2.05 mmol), TPSCl (1.55g, 5.13 mmol) and DMAP (0.626 g 5.13 mmol) were dissolved in dry acetonitrile (25 mL), then cooled to 0 ºC. TEA (0.71 mL) was added dropwise and the reaction mixture was stirred for 12 hours at room temperature. Ammonia gas was bubbled into the reaction mixture for 30 min at 0 °C, then the mixture was stirred for 15 minutes at room temperature. The volatiles were removed

under vacuum and the residue was partitioned between EtOAc and H_2O. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography (5% EA / DCM) to give a mixture of **21** its partially deprotected derivative **23** as a pale-yellow foam (1.1 g, 73.48% yield). Compounds **21** and **23** were partially separated for analytical purposes. Analytical data for compound **21**: ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 5.71 (d, *J* = 4.0 Hz, 1H, H-1'), 4.53 (br dd, *J* = 4.0, 8 Hz, 1H, H-2'), 4.29 (br dd, *J* = 4. 8 Hz, 1H, H-3'), 3.97 (m, 1H, H-4'), 3.73-3.65 (m, 2H, H-5'a and H-5'b), 2.16 (s, 3H, 5-CH_3), 0.92-0.86 (4s, 36H, *tert*-BuSi), 0.09-00 (8s, 24H, Si-CH_3). Analytical data for compound **23**: ^{1}H NMR(400 MHz, DMSO-d_6) δ ppm: 7.87 (s, 1H, N-OH), 7.79 (br s, 1H, NH), 7.18 (br s, 1H, NH), 5.70 (d, *J* = 5.2 Hz, 1H, H-1'), 4.47 (br dd, *J* = 4.8 Hz, 1H, H-2'), 4.29 (br dd, *J* = 4.0 Hz, 1H, H-3') , 3.76 (m, 1H, H-4'), 3.64-3.56 (m, 2H, H-5'a, H-5'b), 2.01 (s, 3H, 5-CH_3), 0.88 (m, 28H, *tert*-BuSi), 0.08-0.01 (6s, 18H, Si-CH_3).

1-(β-D-Ribofuranosyl)-2-hydroxylimino-6-aza-5-methylcytosine (9). 1*M* solution of TBAF in THF (6 mL) was added dropwise to a solution of the mixture of **21** and **23** (1g, 1.369 mmol) in dry THF (15 mL) and the reaction mixture was stirred for 3 hours at room temperature. The solvent was removed under reduced pressure and the residue was purified by DOWEX 50 RESIN to give compound **9** (0.22 g, 59.29% yield) as a yellow foam; UV-vis (MeOH) λ_{max} 325nm, 240 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 7.82 (br s, 1H, NH, exchangeable with D_2O) 5.75 (br s, 2H, NH and OH, exchangeable with D_2O), 5.63 (d, *J* = 4.0 Hz, 1H, H-1'), 4.17 (m, 1H, H-2'), 3.92 (m, 1H, H-3'), 3.72 (m, 1H, H-4'), 3.51-3.38. (m, 2H, H-5'a, and H-5'b), 2.01 (s, 3H, CH_3);APT ^{13}C NMR (100 MHz, DMSO- d_6) δ ppm: 152.77 (C-4), 149.01 (C-2), 130.56 (C-5), 90.97 (C-1'), 84.11 (C-2'), 71.95 (C-3'), 70.82 (C-4'), 62.58 (C-5'), 19.19 (CH_3); Anal cald For $C_9H_{15}N_5O_5$: C, 39.56; H, 5.53; N, 25.63. Found: C, 39.44; H, 5.65; N, 25.74.

2,2',3',5'-Tetra-*O*-*tert*-butyldimethylsilyl-β-D-ribo-furanosyl-6-aza-2-hydoxyimino -5-methyl-4-thiouridine (22). To a mixture of compound **20** (1.5g, 2.05 mmol), and Lawesson reagent (0.829 g, 2.1 mmol) in dry toluene (50 mL) was heated at reflux temperature overnight. The mixture was cooled down to room temperature and the volatiles were removed under vacuum. The residue was purified by silica gel column chromatography (eluate: 1% ethyl acetate/hexane) to give compound **22** (1 g, 65.27% yield) as a dark yellow syrup: UV-vis

(MeOH) λ_{max} 230 (shoulder), 275, 320 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 10.04 (s, 1H, NH), 5.83 (d, *J* = 4.4 Hz, 1H, H-1'), 4.44 (br dd, *J* = 4.4Hz, 1H, H-2'), 4.30 (br dd, 1H, H-3'), 3.94 (m, 1H, H-4'), 3.73-3.64 (m, 2H, H-5'a,b), 2.28 (s, 3H, 5-CH_3), 0.90 (m, 36H, t-Bu-Si), 0.05-0.2 (m, 24H, Si-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 180.10 (C=S), 143.41 (C=N), 140.85 (C=N), 89.34 (C-1'), 84.20 (C-2'), 73.65 (C-3'), 71.88 (C-4'), 62.76 (C-5'), 53.57 (t-Bu-Si), 51.05 (t-Bu-Si), 29.85 (t-Bu-Si), 26.40 (t-Bu-Si), 26.05 (t-Bu-Si), 26.00 (t-Bu-Si), 25.88 (t-Bu-Si), 20.06 (5-CH_3), 18.56 (Si-$\underline{C}Me_3$), 18.21 (Si-$\underline{C}Me_3$), 1.17 (CH_3-Si), 0.14 (CH_3-Si), -4.27 (CH_3-Si), -4.36 (CH_3-Si), -4.46 (CH_3-Si), -4.57 (CH_3-Si), -5.13 (CH_3-Si), -5.14 (CH_3-Si), -5.27 (CH_3-Si).

1-(β-D-Ribofuranosyl)-6-aza-2-hydoxyimino-5-methyl-4-thiouracil (8). 1*M* solution of TBAF in THF (6.4 mL) was added dropwise to a solution of compound **22** (1g, 1.338 mmol) in dry THF (15 mL) and the reaction mixture was stirred for 3 hours at room temperature. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (eluate: 8:1:1:1 ethyl acetate/ ethanol / acetone/ H_2O) to give compound **8** (0.302 g, 77.92% yield) as a yellow solid: M. p.,160-162 ºC; UV-vis (MeOH) λ_{max} 325 nm; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.66 (s, 1H, NH, exchangeable with D_2O), 9.79 (s, 1H, N=OH, exchangeable with D_2O), 5.62 (d, *J* = 3.6 Hz, 1H, H-1'), 5.24 (br s, 1H, 2'-OH, exchangeable with D_2O), 4.98 (br d, 1H, 3'-OH, exchangeable with D_2O), 4.66.(br s, 1H, 5'-OH, exchangeable with D_2O), 4.26 (br s, 1H, H-2'), 4.01 (br dd, 1H, H-3'), 3.77 (m, 1H, H-4'), 3.51 (m, 1H, H-5'a), 3.39 (br m, 1H, H-5'b,) 2.17 (s, 3H, CH_3); APT ^{13}C NMR (100 MHz, DMSO- d_6) δ ppm: 180.93 (C=S), 140.62 (C=N), 139.91 (C=N), 90.70 (C-1'), 84.69 (C-2'), 72.08 (C-3'), 70.53 (C-4'), 62.22 (C-5'), 20.05 (CH_3); IR (ATR, cm^{-1}): 3375.17 (νOH), 3225.83 (ν OH), 2952. 91 (ν OH), 2908.38 (ν NH), 1628.64 (νC=N), 1551.45 (νC=S); EI-Mass *m/z* 290.88 [M^+]; Anal cald for $C_9H_{14}N_4O_5S$: C, 37.24; H, 4.86; N, 19.30. Found: C, 37.39; H, 4.94; N, 19.21.

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