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Comparative inhibitory activity of 3′- and 5′-functionalized nucleosides on ribonuclease A

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ABSTRACT

Modified nucleosides, molecules, functionalized with various polar groups at different positions have been synthesized to rationalize the impact of structural modification on their inhibitory activity. Agarose gel and precipitation assays indicate their improved inhibitory activity on ribonuclease A (RNase A). Kinetic experiments clearly categorize them as competitive inhibitors of RNase A with improved inhibition constant (K_i) values (37 ± 9, 67 ± 6, and 193±7 μM for compounds **10**, **3**, and **7**, respectively). The preferential hydrogen bonding network formation between His-12 and His-119 of RNase A with the polar carboxylic and amino groups of these compounds has been evidenced from the docking studies. The relationship between structural modifications and inhibitory activity of these compounds is further justified in terms of energetics using *PEARLS*.

1. Introduction

Mammalian ribonucleases are non-cytosolic endonucleases[1] that become cytotoxic when absorbed in cells.[2] A growing number of proteins of this superfamily, e.g., eosinophil-derived neurotoxin (EDN),[3] eosinophil cationic protein (ECP),[4] angiogenin,[5] bovine seminal RNase A[6] are found to have an enhanced effect on many disease processes.[7] Therefore, inhibitor designing for ribonucleases is a potential field for synthetic chemists.

Ribonuclease A (RNase A) is the most explored model protein of the ribonuclease superfamily. The ribonucleolytic site of RNase A comprises of different subsites, namely P_0—P_n for phosphate group recognition of RNA; R_0—R_n for the recognition of ribose moiety and B_0—B_n for the recognition of nucleobases[8,9] (Fig. 1). RNase A cleaves ribonucleic acid (RNA) via two steps. The first step is the transphosphorylation step where His-12 and His-119 act as a base and acid, respectively.[10] However, for the hydrolysis step (second step) His-12 and His-119 play a completely opposite role than in the transphosphorylation step.[10]

There are a large number of reported nucleotidic inhibitors of RNase A. During the interaction of these inhibitors with RNase A the acidic phosphate or pyrophosphate groups alter the pK_a values

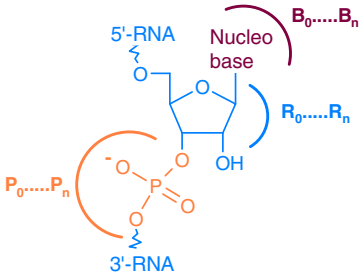

Figure 1. Ribonucleolytic subsites of RNase A.

of His-12 and His-119 which are important members of P_1 site[11–13] and the crystal structures show their direct interactions with the P_1 site residues.[14–16] However, the high negative charge of these nucleotidic inhibitors is one of their major limitations.[17,18] Therefore, to increase the lipophilicity different modified nucleosides have been synthesized which showed moderate inhibitory activity towards RNase A.[19–23] These molecules also show their ability to perturb the active site acid–base equilibrium like the other nucleotidic inhibitors.[22,24]

Herein we report the synthesis of several modified nucleosides which are functionalized by various non-phosphate polar groups at their different positions. Adenosine and thymidine molecules are functionalized with carboxyl groups at their 5′-position and then their inhibitory activities are compared with the 3′-carboxyl functionalized thymidines. This comparison is expected to provide

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valuable information regarding the positional functionalization of nucleoside molecules for the designing of potent nucleosidic inhibitors of ribonuclease superfamily enzymes. On the other hand nucleosides molecule with more than one polar group have also been prepared and compared with others to estimate the importance of ionic interaction for the further refinement of inhibitor designing.

2. Results and discussion

2.1. Synthesis of modified nucleosides

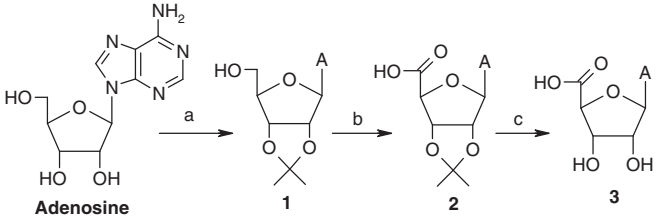
The selection of protecting groups was an important underlying feature for the functionalization of nucleosides at the required position. For selective oxidation of the 5′-hydroxyl group, acid-labile isopropylidene protected adenosine **1** was synthesized by reacting adenosine with 2,2-dimethoxypropane in the presence of catalytic amount of *p*-toluene sulphonic acid.[25] The 5′-OH group was oxidized by sodiumperiodate ($NaIO_4$) to obtain compound **2**. The isopropylidene group of **2** was removed by 60% trifluoroacetic acid (TFA) in DCM to yield the desired compound **3** (Scheme 1). Similarly, 5′-carboxylated analog of thymidine **7** was synthesized from tritylated thymidine **4**. Since the susceptibility of silyl-ether linkage towards lewis acid ($ZrCl_4$)[26] was reported earlier, we protected the 3′-hydroxyl group of **4** by *tert*-butyldimethylsilyl (TBDMS) group; we opined that TBDMS group would be removed simultaneously during the oxidation of 5′-OH of compound **6** using a Lewis acid $RuCl_3$ (Scheme 2). However, in this case we replaced $NaIO_4$ with $K_2S_2O_8$ due to the presence of the pyrimidine moiety.[27]

Compound **10** functionalized with both carboxyl and amino groups was synthesized from aminothymidine **8** (Scheme 3). In the first step Boc-Asp(OBzl)–OH was coupled with the 3′-amino group of **8** using dicyclohexylcarbodiimide (DCC) as a coupling agent.[28] Thereafter, the benzyl group of **9** was removed using Pd/C under H_2 atmosphere and finally without further purification the acid-labile trityl and Boc groups were removed by TFA in DCM to afford compound **10**. Synthesis of **Ser-aT**, **Oxa-aT**, and **Suc-aT** was reported earlier.[22,23]

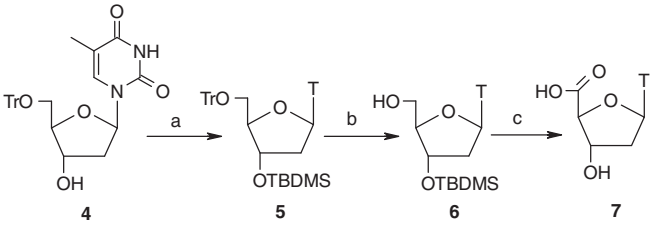
2.2. Biological evaluation

The inhibitory activities of compounds **3**, 7, and **10** were qualitatively checked by an agarose gel-based assay. The maximum intensity of lane 1 indicated the least possible degradation of RNA, whereas the lowest intensity of lane 2 showed the maximum possible degradation of RNA in presence of RNase A. However, the greater intensities of lanes 3, 4, and 5 compared to lane 2 indicated the inhibitory activity of synthesized compounds (Fig. 2) and also qualitatively identified compound **10** as the most potent inhibitor of RNase A.

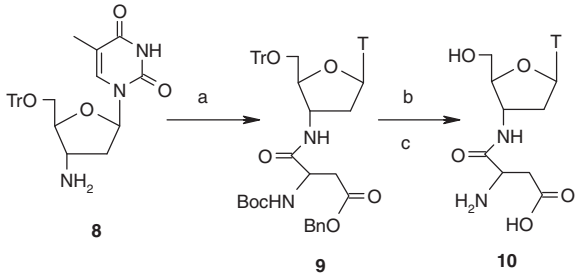
After getting the indication about the inhibitory activity of compounds **3, 7**, and **10** we quantitatively estimated their inhibitory potency by the precipitation assay. This assay showed compounds



Scheme 1. Synthesis of 5′-carboxy adenosine. Reagents and conditions: (a) 2,2-dimethoxypropane, *p*-TSA, DMF, 70 °C, 8 h; (b) $NaIO_4$, $RuCl_3$, $H_2O/CH_3CN/CCl_4$ (3:2:2), rt, 3 h; (c) 60% TFA in DCM, rt, 3 h.



Scheme 2. Synthesis of 5′-carboxy thymidine. Reagents and conditions: (a) TBDMSCl, imidazole, Py, rt, 10 h; (b) 10% TFA in DCM, rt, 2 h; (c) $K_2S_2O_8$, $RuCl_3$, 1 N KOH, rt, 3.5 h.



Scheme 3. Synthesis of compound **10**. Reagents and conditions: (a) Boc-Asp(OBzl)–OH, DCC, DMAP,DMF, 0 °C to rt, 30 h; (b) Pd/C, H_2, MeOH, 50 °C, 10 h; (c) 60% TFA in DCM, rt, 4 h.

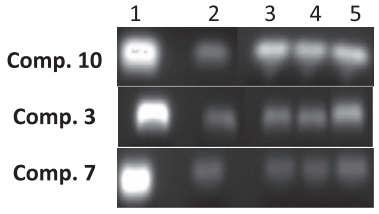


Figure 2. Agarose gel-based assay for the inhibition of RNase A by the modified nucleosides. RNase A concentration: 0.66 μM and concentration of all compounds 1.9 mM. Lane 1: tRNA; lane 2: tRNA and RNase A; lanes 3, 4, and 5: tRNA and RNase A with increasing concentration of inhibitors.

10, **3**, and **7** inhibited the ribonucleolytic activity of RNase A by 47.65 ± 2.6%, 32.25 ± 1.9%, and 23.54 ± 2.1%, respectively (Fig. 3). From this data it was clear that compounds with two polar ionizable groups (compound **10**) was a more promising inhibitor than **3** and **7** having only one polar ionisable group each.

To elucidate the mode of inhibition of these synthesized compounds kinetic experiments were conducted. We used cyclic cytidinemonophosphate (cCMP) as the substrate of RNase A for this kinetic experiment. The reciprocal of the initial velocity data was plotted against the inhibitor concentration. From the plots of

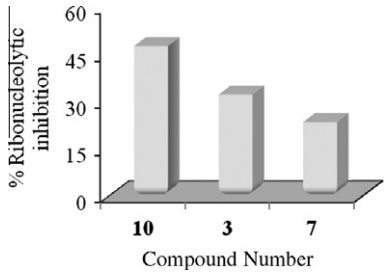


Figure 3. Inhibition of ribonucleolytic activity by modified nucleosides (**10**, **3**, and **7**). Compounds and RNase A concentrations are 2.5 mM and 0.6 μM, respectively.

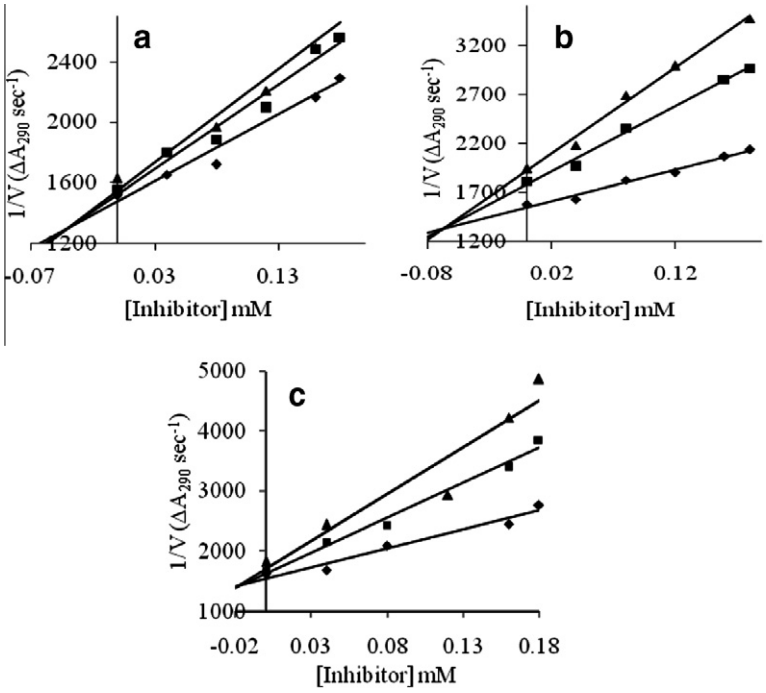
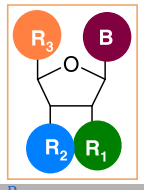
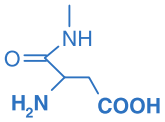
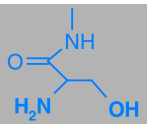
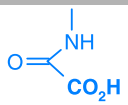
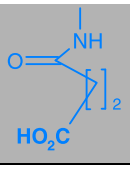


Figure 4. Dixon plots for RNase A inhibition by (a) compound **10**: substrate concentrations: 0.33 mM (♦), 0.27 mM (■), and 0.2 mM (▲); (b) compound **3**: substrate concentrations: 0.26 mM (♦), 0.21 mM (■), and 0.16 mM (▲); and (c) compound **7**: substrate concentrations: 0.25 mM (♦), 0.2 mM (■), and 0.15 mM (▲). The RNase A concentration: 10 μM.

kinetic experiment the inhibition constant values (K_i) were found 37 ± 9, 67 ± 6, and 193 ± 7 μM for compound **10**, **3**, and **7**, respectively. However, the nature of the plots clearly depicted that these compounds inhibit the ribonucleolytic activity of RNase A in a competitive fashion (Fig. 4).

Table 1
RNase A inhibition constant (K_i) values of modified nucleosides with different functionalities



Compd	R_1	R_2	R_3	B	K_i (μM)
10	–H		$-CH_2OH$	T	37 ± 9
Ser-aT	–H		$-CH_2OH$	T	80 ± 3[22]
3	–OH	–OH	$-CO_2H$	A	67 ± 6
7	–H	–OH	$-CO_2H$	T	193 ± 7
Oxa-aT	–H		$-CH_2OH$	T	132 ± 2[23]
Suc-aT	–H		$-CH_2OH$	T	981 ± 2[23]

The K_i values of these compounds clearly showed the effect of structural modification of nucleosides by polar groups at different positions; the addition of extra ionisable groups influenced their inhibitory activity towards RNase A (Table 1). A comparison of the inhibitory activities of **3** and **7** established that the carboxyl functionalized adenosine analog **3** was a better inhibitor than the thymidine analog **7**. However, the K_i values of **Oxa-aT** and **7** (Table 1) reflected the fact that perhaps the functionalization of the 3′-position of thymidine with a polar group was a better option than having the same group at the 5′-position. On the other hand, for **Ser-aT** it was found that the presence of an additional hydroxyl group along with the ionisable amino group made it the most potent inhibitor (K_i = 80 ± 3 μM) among the other members of that series as reported earlier.[22] This observation highlighted the importance of the presence of more than one polar group for the better inhibitory activity. For compound **10**, functionalized with one amino and one carboxyl group (more polar than the hydroxyl group) the K_i value was found to be lowered to a large extent compared to **Ser-aT** (80 ± 3 μM). Now for **Suc-aT** (K_i = 981 ± 2 μM[23]), there was no additional polar group compared to the structure of compound **10** and the absence of an additional amino group was responsible for its poor inhibitory activity compared to **10**. However, it is also probable that the ionized carboxylate anion of **10** interacted with the protonated amino acid residues of P_0—P_n subsites electrostatically which are not possible in the case of amino-alcohol containing compound **Ser-aT**. This implied combination of strong polar groups was also another important structural requirement for the designing of inhibitors of ribonuclease superfamily enzymes.

2.3. Theoretical investigation

The possible mode of orientation of the modified nucleosides with RNase A was elucidated with the help of docking studies.

For compound **3** the carboxylic group at the 5′-position was found within the hydrogen bonding distance of His-12. Along with that it also formed a hydrogen bonding network with Gln-11. Interestingly the 2′-OH was also in close proximity of His-119 and its adenine moiety was found to form a hydrogen bonding framework with Lys-41, Val-43, and Phe-120. In case of compound **7**, we found almost the same binding pattern as in case of **3**. Here the carboxylic group formed hydrogen bonding network with Gln-11, His-12, and Phe-120. Unlike compound **3** the 3′-OH of compound **7** was found near the hydrogen bonding distance with His-119. However, the thymine moiety of **7** was found to form a hydrogen bonding network with Lys-7 and Gln-11 (Fig. 5). Form the docked conformation of **Oxa-aT**[23] we found the carboxylic group well interacted with both His-12 and His-119 residues but the pyrimidine moiety was situated quite far from its assigned recognition site. Probably it is one of the important reasons for the better inhibitory activity of compound **3** compared to **Oxa-aT**.

Similarly comparing the docking conformation of compound **10** with **Ser-aT**[22] and **Suc-aT**[23] it was found that the carboxylic group of **10** was within hydrogen bonding distance of His-12. On the contrary, for **Ser-aT** its amino group preferentially interacted with His-119 and for **Suc-aT** its carboxy group interacted with both His-119 and His-12. The 3′-amide nitrogen was also found within hydrogen bonding distance of His-119 for compound **10** similar to that of **Ser-aT**. For compound **10** it was found that the free amino group and 5′-OH were situated within hydrogen bonding distance of Phe-120 and Val-118, respectively (Fig. 6) but the hydrogen bonding interaction with Phe-120 was completely absent for both **Ser-aT** and **Suc-aT**.

Apart from the docking conformations of the modified nucleosides, individual interaction energies were calculated using *PEARLS*[29] to understand how the structural modifications influence their inhibitory activity in terms of energetics. In all the docked conformations of compounds **3**, **7**, and **Oxa-aT** it was found that the carboxylic moiety preferentially interacted with the P_1 site residues and placed their nucleobase part accordingly. Therefore, van der Waals interaction energy for these compounds was expected to play the determining role to incorporate their nucleobases within the protein framework. The van der Waals interaction energy of **3**, **7**, and **Oxa-aT** were found −6.05, −4.69, and −5.01 kcal/mol, respectively, and these values clearly indicated among these three modified nucleosides adenine moiety binds more favorably near the B_1 site rather than the thymine moiety which in turn influenced its inhibitory activity.

However, for compounds **10**, **Ser-aT**, and **Suc-aT** the modification was made only at the 3′-position of thymidine molecule. The only difference among their structure was the combination of their polar groups. The only structural difference between compound **10**

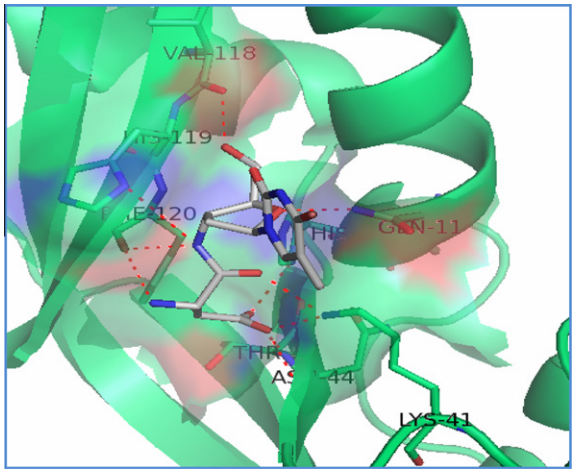


Figure 6. Docked conformations of compound **10** (gray) with RNase A (1FS3).

and **Ser-aT** was the replacement of one –OH group (for **Ser-aT**) by one –COOH group (for compound **10**). Similarly, the structural difference between **10** and **Suc-aT** was only the incorporation of an additional amino group next to the amide carbon of the amide bond at the 3′-position. Therefore, it was well anticipated that for this sets of compounds the electrostatic interaction energy played the decisive role to determine their inhibitory potency. The electrostatic interaction energy for **10**, **Ser-aT**, and **Suc-aT** were −2.01, −0.48, and 1.47 kcal/mol, respectively. These energy values were also in well agreement of our assumption.

The K_i values of compound **7** and **Oxa-aT** (193 ± 7 and 132 ± 2 μM, respectively) showed modification of thymidine by carboxy functionalization at the 3′-position was a better option than at the 5′-position. However, adenosine was the more preferred alternative than thymidine for carboxy functionalization at the 5′-position as reflected from their K_i values. Similarly, K_i value of compound **10** (37 ± 9 μM) indicated the presence of additional polar group was an essential criteria for the improvement of the inhibitory activity. The comparative K_i values of **10**, **Ser-aT**, and **Suc-aT** demonstrated that the polar functionalization closer to the main nucleosidic moiety was also an important feature for the evolution of more potent inhibitor devoid of any phosphate or pyrophosphate group. These findings reflected that chemical modifications at different position of nucleosides can cause a substantial alternation of their inhibitory activity. The chemical modifications of nucleosides molecules can also generate inhibitors with better potential than the nucleotidic inhibitors.[30,31] The docking and *PEARLS* calculation dictated the role of hydrophobic

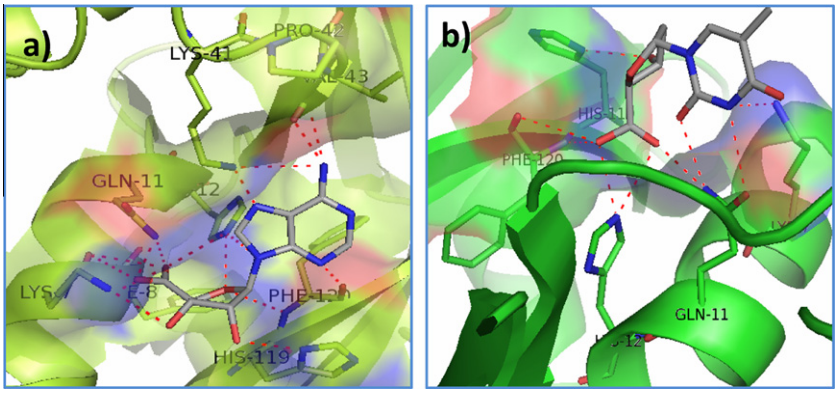


Figure 5. Docked conformations of (a) compound **3** (gray) and (b) compound **7** (gray) with RNase A (1FS3).

and electrostatic interactions for the designing of nucleosidic inhibitors of RNase A.

3. Experimental section

3.1. Materials

Bovine pancreatic RNase A, yeast tRNA, 2′,3′-cCMP, human serum albumin (HSA) were purchased from Sigma–Aldrich. All other reagents were purchased from SRL India. Column chromatographic separations were performed using silica gel (60–120 and 230–400 mesh). HPLC separations were performed by Shimadru Prominence Series HPLC system. Luna 10 μm C18 column (100A) was used for the separation. Solvents were dried and distilled following the standard procedures. TLC was carried out on pre-coated plates (Merck silica gel 60, f_{254}), and the spots were visualized with UV light or by charring the plates dipped in 5% H_2SO_4–MeOH solution or 5% H_2SO_4/vanillin/EtOH or 5% ninhydrine in MeOH solution. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker NMR spectrometer (δ scale). Acetonitrile was used as a reference in case of NMRs taken in D_2O solvent. UV–vis measurements were made using a Perkin Elmer UV–vis spectrophotometer (Model Lambda 25). Concentrations of the solutions were determined spectrophotometrically using the following data: $\varepsilon_{278.5}$ (RNase A),[32] ε_{268} (2′,3′-cCMP)[31] are 9800 and 8500 M^{-1} cm^{-1}, respectively. High resolution mass spectra were recorded on Micromass LCT.

3.2. Synthesis of modified nucleosides

3.2.1. 2′,3′-Isopropylidene adenosine (1)

To the stirred solution of adenosine (0.25 g, 0.9 mmol) in anhydrous DMF (6 ml), 2,2-dimethoxypropane (0.6 ml, 4.5 mmol) and anhydrous *p*-toluenesulphonic acid (0.04 g, 0.2 mmol) were added and the mixture was stirred at 70 °C for 8 h (TLC) under the N_2 atmosphere. The reaction mixture was concentrated under vacuo and the residue was stirred with saturated aq $NaHCO_3$ solution for another 6 h. The organic portion was extracted with EtOAc and all the organic portions were evaporated under reduced pressure. Crude residue thus obtained was purified over silica gel to afford compound **2** (0.21 g, 73%). white solid. ^{1}H NMR: (d_6-DMSO): δ 1.30 (s, 3H), 1.52 (s, 3H), 3.48–3.56 (m, 2H), 4.19 (q, J = 4.4, 6.8 Hz, 1H), 4.94 (dd, J = 2.6 Hz, 1H), 5.23 (t, J = 5.6 Hz, 1H), 5.32 (q, J = 3.2, 6.4 Hz, 1H), 6.10 (d, J = 2.8 Hz, 1H), 7.34 (br s, 2H), 8.13 (s, 1H), 8.32 (s, 1H).

3.2.2. 5′-Carboxy-2′,3′-isopropylidene adenosine (2)

Compound **1** (0.74 g, 2.4 mmol) was dissolved in a ternary solution mixture of H_2O, CH_3CN, and CCl_4 (3:2:2; 2.6 ml). To this solution sodium periodate (1 g, 4.6 mmol) and $RuCl_3{\cdot}3H_2O$ (0.008 g) were added and the mixture was stirred vigorously at room temperature for 3 h (TLC). The reaction mixture was concentrated under vacuo. Crude residue thus obtained was purified over silica gel to afford compound **2** (0.41 g, 52%). ^{1}H NMR: (d_6-DMSO): δ 1.34 (s, 3H), 1.51 (s, 3H), 4.70 (s, 1H), 5.52 (s, 2H), 6.41 (s, 1H), 8.51 (s, 1H), 8.55 (s, 1H), 10.08 (s, 1H).

3.2.3. 5′-Carboxy adenosine (3)

A solution of compound **2** (0.3 g, 0.9 mmol) in 60% TFA in DCM (30 ml) was stirred at room temperature for 3 h (TLC). The reaction mixture was concentrated under vacuo. Crude residue thus obtained was purified over silica gel to afford compound **3** (0.16 g, 63%). ^{1}H NMR: (D_2O): δ 3.37 (br s, 1H), 4.12 (s, 2H), 6.07 (d, J = 6 Hz, 1H), 8.06 (s, 1H), 8.53 (s, 1H). ^{13}C NMR (D_2O): δ 73.9, 74.5, 85.0, 86.6, 119.1, 140.4, 149.2 (C), 152.7, 155.5 (C), 176.5 (C). HRMS (ESI^+): m/z calculated for $C_{10}H_{11}N_5O_5Na$ $[M+Na]^+$: 304.0652; found: 304.0660.

3.2.4. 5′-*O*-Trityl-3′-*tert*-butyldimethylsilyl thymidine (5)

To the stirred solution of compound **4** (1 g, 2.1 mmol) in anhydrous pyridine (30 ml), *tert*-butyldimethylsilylchloride (1.2 g 7.9 mmol) and imidazole (0.7 g, 10 mmol) were added and the mixture was stirred at room temperature under N_2 atmosphere for 10 h (TLC). Saturated aq $NaHCO_3$ solution was added and the mixture was stirred for another 6 h. The organic portion was extracted with EtOAc and all organic portions were evaporated under reduced pressure. Crude residue thus obtained was purified over silica gel to afford compound **5** (1.2 g, 97%). ^{1}H NMR: ($CDCl_3$): δ 0.04 (s, 3H), 0.02 (s, 3H), 0.83 (s, 9H), 1.49 (s, 3H), 2.19–2.26 (m, 1H), 2.31–2.36 (m, 1H), 3.28 (dd, J = 2.4, 10.4 Hz, 1H), 3.46 (dd, J = 2.8, 10.8 Hz, 1H), 3.97 (d, J = 2.8 Hz, 1H), 4.55 (m, 1H), 6.35 (t, J = 6.4 Hz, 1H), 7.25–7.43 (m, 15H), 7.64 (s, 1H). ^{13}C NMR ($CDCl_3$): δ −4.9, −4.7, 11.9, 17.8 (C), 25.7, 41.5 (CH_2), 63.0 (CH_2), 72.0, 84.8, 86.6, 87.3 (C), 111.0 (C), 127.4, 128.0, 128.6, 135.5, 143.3 (C), 150.2 (C), 154.9 (C).

3.2.5. 3′-*tert*-Butyldimethylsilyl thymidine (6)

Compound **5** (1.25 g, 2 mmol) was stirred with 10% TFA in DCM (5 ml) for 2 h (TLC) at room temperature. The reaction mixture was concentrated under vacuo. Crude residue thus obtained was purified over silica gel to afford compound **6** (0.41 g, 55%). ^{1}H NMR: ($CDCl_3$): δ 0.08 (s, 6H), 0.89 (s, 9H), 1.91 (s, 3H), 2.18–2.24 (m, 1H), 2.32–2.39 (m, 1H), 3.75 (m, 1H), 3.92 (m, 2H), 4.49 (m, 1H), 6.13 (t, J = 6.8 Hz, 1H), 7.36 (s, 1H). ^{13}C NMR ($CDCl_3$): δ −4.9, −4.8, 12.4, 17.9 (C), 25.6, 40.4 (CH_2), 61.8 (CH_2), 71.5, 86.6, 87.5, 110.9 (C), 137.0, 150.4 (C), 164.0 (C).

3.2.6. 5′-Carboxy thymidine (7)

To a stirred solution of compound **6** (0.4 g, 1.1 mmol) in aq KOH (1 N; 8 ml), $K_2S_2O_8$ (1.3 g, 4.8 mmol), and $RuCl_3{\cdot}3H_2O$ (0.004 g) were added and the mixture was stirred vigorously for 3.5 h (TLC) at room temperature. The reaction mixture was neutralized by dil. HCl to pH 7. The mixture was concentrated under vacuo. Crude residue thus obtained was purified over silica gel to afford compound **7** (0.2 g, 69%). ^{1}H NMR: (d_6-DMSO): δ 1.75 (s, 3H), 1.84–1.89 (m, 1H), 2.04–2.08 (m, 1H), 4.20 (s, 1H), 4.37 (d, J = 4 Hz, 1H), 6.28 (q, J = 5.2, 12.8 Hz, 1H), 8.60 (s, 1H), 11.25 (s, 1H). ^{13}C NMR (d_6-DMSO): δ 12.5, 38.8 (CH_2), 74.3, 85.6, 86.4, 109.2 (C), 137.6, 150.7 (C), 164.0 (C), 174.1 (C). HRMS (ESI^+): m/z calculated for $C_{10}H_{12}N_2O_6Na$ $[M+Na]^+$: 279.0588; found: 279.0593.

3.2.7. 3-*tert*-Butoxycarbonylamino-*N*-[5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2-trityloxymethyl-tetrahydro-furan-3-yl]-succinamic acid benzyl ester (9)

A mixture of compound **8** (0.54 g, 1.1 mmol) and Boc-Asp(OBzl)–OH (0.36 g, 1.1 mmol) in anhydrous DMF (10 ml) was cooled to −15 °C. Dicyclohexylcarbodiimide (0.23 g, 1.1 mmol) and *N*-hydroxysuccinamide (0.13 g, 1.1 mmol) were added and the reaction mixture was stirred for 30 h(TLC) at room temperature under N_2 atmosphere. The reaction mixture was filtered and the residue was washed with EtOAc. The filtrate was stirred with saturated aq NH_4Cl solution for another 6 h. The organic portion was then extracted with EtOAc and all the organic portions were evaporated under reduced pressure. Crude residue thus obtained was purified over silica gel to afford compound **9** (0.53 g, 60%). ^{1}H NMR: ($CDCl_3$): δ 1.36 (s, 3H), 1.43 (s, 9H), 2.35–2.45 (m, 2H), 2.69 (dd, J = 5.6, 16.8 Hz, 1H), 2.92 (dd, J = 4, 16.4 Hz, 1H), 3.41 (m, 2H), 3.92 (s, 1H), 4.49 (br s, 1H), 4.73 (m, 1H), 5.09 (m, 2H), 5.70 (d, J = 8.4 Hz, 1H), 6.41 (m, 1H), 7.23–7.42 (m, 20H), 7.52 (br s, 1H), 7.60 (s, 1H), 9.47 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 11.6, 28.2, 36.3 (C), 37.9 (C), 50.6, 63.8 (CH_2), 66.9 (CH_2), 80.8 (C), 84.2, 84.5, 87.4 (C), 111.7 (C), 127.2, 128.0, 128.2, 128.3,

128.5, 128.7, 135.2 (C), 135.3, 143.2 (C), 151.0 (C), 163.7 (C), 170.8 (C), 171.3 (C). HRMS (ESI^+): *m/z* calculated for $C_{45}H_{48}N_4O_9Na$ $[M+Na]^+$: 811.3314; found: 811.3319.

3.2.8. 3-Amino-*N*-[2-hydroxymethyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-succinamic acid (10)

To a stirred solution of compound **9** (0.3 g, 0.4 mmol) was dissolved in dry methanol (10 ml). Then to this stirred solution Pd/C (10%) was added and the mixture was stirred for 10 h (TLC) at 50 °C under H_2 atmosphere. The reaction mixture was filtered and the residue was washed with MeOH. The filtrate thus obtained was evaporated under reduced pressure. The crude residue was dissolved in 60% TFA in DCM (5 ml) to this reaction product and stirred for 4 h (TLC) at room temperature. Volatile materials were removed under vacuo and the reaction product thus obtained was purified using HPLC to afford compound **10** (0.06 g, 52%). ^1H NMR: (d_4-MeOH): δ 1.89 (s, 3H), 2.28–2.38 (m, 2H), 2.76–2.92 (m, 2H), 3.31 (s, 3H), 3.73 (dd, J = 2.8, 12 Hz, 1H), 3.84–3.90 (m, 3H), 4.20–4.24 (m, 1H), 4.52 (q, J = 6.4 Hz, 1H), 6.22 (t, J = 6 Hz, 1H), 7.88 (s, 1H). ^{13}C NMR (D_2O): δ 11.0, 35.3 (CH_2), 37.2 (CH_2), 49.0, 49.6, 60.9 (CH_2), 84.4, 84.9, 110.1 (C), 136.5, 150.9 (C), 164.9 (C), 169.3 (C), 170.0 (C). HRMS (ESI^+): *m/z* calculated for $C_{14}H_{21}N_4O_7$ $[M]^+$: 357.1405; found: 357.1412.

3.3. Agarose gel-based assay

Inhibition of RNase A by all the synthesized modified nucleosides was checked qualitatively by the degradation of tRNA in an agarose gel. In this method, 20 μl of RNase A (0.66 μM) was mixed with 10, 15, and 20 μl of the compounds (1.9 mM) to a final volume of 50 μl and the resulting solutions were incubated for 6 h at 37 °C. Then 20 μl aliquots from the incubated mixtures were mixed with 20 μl of tRNA solution (5.0 mg/ml) and 10 μl of sample buffer (containing 10% glycerol and 0.025% bromophenol blue). The mixture was then incubated for another 30 min. A volume of 15 μl from each solution were extracted and loaded onto a 1.1% agarose gel. The gel was run in 0.04 M Tris-acetic acid-EDTA (TAE) buffer (pH 8.0). The undegraded tRNA was visualized by ethidium bromide staining under UV light. Gel images were captured on a KODAK Gel Logic 200 imaging system.

3.4. Precipitation assay

Inhibition of the ribonucleolytic activity of RNase A was quantified by the precipitation assay as described by Bond.[33] In this method 10 μl of RNase A (0.6 μM) was mixed with 50 μl of each nucleoside-dibasic acid conjugate (2.5 mM) to a final volume of 100 μl and incubated for 2 h at 37 °C. A 20 μl aliquot of the resulting solutions from the incubated mixtures were then mixed with 40 μl of tRNA (5 mg/ml), 40 μl of Tris–HCl buffer of *pH* 7.5 containing 5 mM EDTA and 0.5 mg/ml HSA. After incubation of the reaction mixture at 25 °C for 30 min, 200 μl of ice-cold 1.14 (N) perchloric acid containing 6 mM uranyl acetate was added to quench the reaction. The solution was then kept in ice for another 30 min and centrifuged at 4 °C at 12,000 rpm for 5 min. A 100 μl aliquot of the supernatant was taken and diluted to 1 ml. The change in absorbance at 260 nm was measured and compared to a control set.

3.5. Inhibition kinetics

The inhibition of RNase A by compounds **10**, **3**, and **7** was assessed individually by a spectrophotometric method as described by Anderson et al.[31] The assay was performed in oligovinylsulfonic acid free[34] 0.1 M Mes-NaOH buffer, *pH* 6.0 containing 0.1 M NaCl using 2′,3′-cCMP as the substrate. The inhibitor concentration was ranged from 0 to 200 μM and the substrate concentration was used from 150 to 330 μM. The RNase A concentration was 10 μM. The inhibition constants (K_i) were determined from initial velocity data. The reciprocal of initial velocity was plotted against the inhibitor concentration (Dixon Plot) according to the equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]K_i}[I] + \frac{1}{V_{max}}\left[1 + \frac{K_m}{[S]}\right]$$

where, v is the initial velocity, [S] is the substrate concentration, [I] is the inhibitor concentration, K_m is the Michaelis constant, K_i is the inhibition constant and V_{max} the maximum velocity.

3.6. Docking studies of the compounds with RNase A (1FS3) and their energetics

The crystal structure of RNase A (PDB entry 1FS3)[35] was downloaded from the Protein Data Bank.[36] As the kinetic experiments were performed at *pH* 6 therefore, we assumed that the ligand molecules with carboxylic functionality existed in their anionic form and for those with amino group existed in their cationic form. On the basis of this assumption we generated the 3D structures of the modified nucleosides were generated by Sybyl6.92 (Tripos Inc., St. Louis, USA) and their energy-minimized conformations were obtained with the help of the TRIPOS force field using Gasteiger-Hückel charges with a gradient of 0.005 kcal/mol. The *FlexX* software as part of the SYBYL suite was used for docking of the synthesized compounds with RNase A. *PyMol*[37] was used for visualization of the docked conformations. The ligand–receptor interaction energies of the docked structures for protein–ligand complexes was computed using *PEARLS*.[29]

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Supplementary data

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