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3′-Oxo-, amino-, thio- and sulfone-acetic acid modified thymidines: Effect of increased acidity on ribonuclease A inhibition

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ABSTRACT

A family of 3′-functionalized thymidines carrying XCH_2COOH (X = O, NH, S, SO_2) groups has been designed as inhibitors of RNase A. This is because it is possible to manipulate the overall acidity of this new class of nucleic 'acids' by changing X from oxygen to the SO_2 group in the series. It is also expected that the acyclic nature of the XCH_2COOH group would provide enough flexibility to the –COOH group to have maximum interactions with the catalytic subsite P_1 of RNase A. As the $-SO_2CH_2COOH$ substituted derivative showed better potency partially because of the increased acidity of the –COOH group, the inhibitory properties of both 5′-substituted and 3′,5′-disubstituted sulfone acetic acid modified thymidines were investigated. Two $-SO_2CH_2COOH$ groups were incorporated with the expectation of targeting two phosphate binding sites simultaneously. Thus, 3′,5′-dideoxy-3′,5′-bis-*S*-[(carboxymethyl)sulfonyl]thymidine emerged as the best inhibitor in this series with a K_i value of 25 ± 2 μM.

1. Introduction

During the last two decades, the ribonuclease superfamily members[1] have generated significant biomedical interest as their enzymatic activities[2–5] are closely associated with some physiological irregularities.[6–8] The angiogenic and tumor-promoting activities of angiogenin,[9–11] the neurotoxic activities of eosinophil-derived neurotoxin (EDN)[12,13] and the cytotoxic activities[14] of bovine seminal ribonuclease[15] are among the few potent biological activities shown by the members of this family. Further studies have shown that most of these pathological conditions are linked to the ribonucleolytic activity of these proteins[16] and hence, inhibitors of their enzymatic activity could be potent pharmaceutical agents. Bovine pancreatic ribonuclease A (RNase A)[16,17] is often exploited as a model system to understand structure–function relationships of members of this superfamily. All the members are active against single-stranded RNA substrates and catalyze RNA breakdown although they show various degrees of ligand recognition and discrimination.[18,19] In RNase A, several subsites are responsible in RNA binding which are conventionally termed as $P_0 \ldots P_n$, $R_0 \ldots R_n$ and $B_0 \ldots B_n$ corresponding to phosphate, ribose and nucleobases of bound RNA, respectively (*n* indicates the position of the group with respect to the cleaved phosphate phosphodiester bond where *n* = 1) (Fig. 1). In all RNases the subsite P_1 is conserved, whereas subsites B_1 and B_2 on each side of P_1 are partially conserved.[20] Nevertheless, B_1 binds pyrimidines, while B_2 has a strong base preference for purines. Like all other ribonucleases, the presence of the His12-Lys41-His119 catalytic

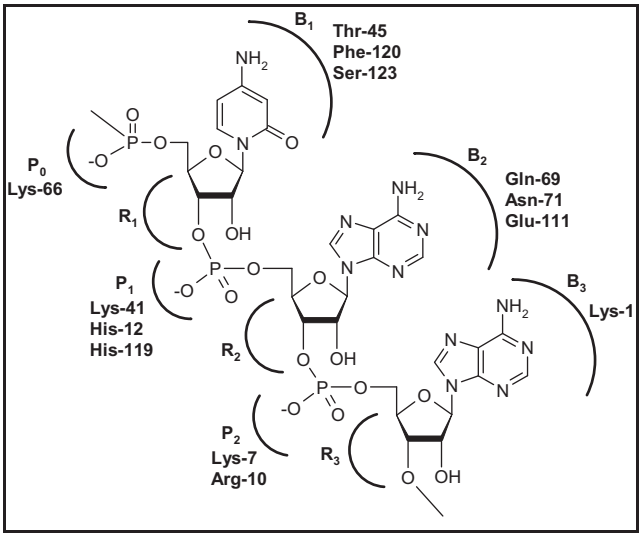

Figure 1. Schematic representation of active clefts of RNase A where B_n, R_n, and P_n are nucleobase-, ribose-, and phosphate-binding subsites, respectively, and labeled with their constituent residues.

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triad of P_1 subsite in RNase A is essential for its ribonucleolytic activity.[16,21]

The development of substrate mimics is a good strategy for the search of a competitive type of inhibitor of an enzyme. Most of the reported potent inhibitors and mechanism based inactivators[22] of RNase A are thus phosphate or pyrophosphate based nucleotide molecules.[20,23–25] The utility of these nucleotides as inhibitors is, however, limited. The free phosphate groups are susceptible to dephosphorylation by phosphatases which markedly diminish their potency as the anionic phosphate groups plays an important role in binding with the active site of RNase A.

Moreover, the polyionic nature of these inhibitors hinders their transport across the cell membrane.[26] To overcome these problems, the replacement of the phosphate group by a carboxylic acid group is considered as an alternative route. At physiological pH, inhibitors carrying acidic groups would exist in the deprotonated form and therefore interact electrostatically with the protonated histidine groups present at the P_1 subsite to perturb the protonating/deprotonating environment of this subsite.[27,28] This in turn would affect the ribonucleolytic activity of RNase A.[29] Few modified mononucleosidic inhibitors containing carboxylic acid group are reported where this acid group is linked through amide linkage[30,31] or by cyclic alkyl amino group[28,32,33] with the backbone of pyrimidine nucleosides. Modified dinucleosides where the phosphate group is replaced with sulfonamide[34] and amide group[35] have also been reported as inhibitors of RNase A. Again, nucleobases tethered with polyphenols and bridged with a triazole moiety have been reported to act as inhibitors of RNase A.[36] Recently triazole pyrimidine nucleosides have emerged as RNase A inhibitors.[37]

It is clearly evident from the earlier experiments carried out in our laboratory that modified nucleosides functionalized with carboxylic group at different positions of the sugar residues as well as dinucleosides having a carboxylic group attached to the internucleoside linkage almost always perform as better inhibitors of RNase A.[35] We therefore selected a family of 3′-functionalized thymidines carrying XCH_2COOH (X = O, NH, S, SO_2) groups. We argued that in this series we would be able to manipulate the overall acidity of this new class of nucleic 'acids' carrying a carboxylic group instead of phosphate by changing X from oxygen to the SO_2 group because it is known that in the series $MeSCH_2COOH$ (pK_a = 3.81), $MeOCH_2COOH$ (pK_a = 3.55), $MeSO_2CH_2COOH$ (pK_a = 2.31) and $MeNHCH_2COOH$ (NH_2-pK_a = 10.2; COOH-pK_a = 2.36),[38,39] acidity of the carboxylic group depends significantly on the nature of X. Since the overall nature of the P_1 site is positively charged at physiological pH,[28,29] a carboxylic group with a pK_a of 2.31 should be able to anchor more efficiently with the P_1 subsite of RNase A. This study is expected to establish that even in the case of weak acids, the efficiency of inhibition of modified nucleosides would vary with the varying pK_a values of the acidic group attached to the sugar moiety of a nucleoside.

The crystal structure of 3′-isonipecotic acid modified *ara*-uridine with RNase A reveals that two inhibitors are bound to the peripheral binding sites of RNase A in a different fashion.[40] In one case, the uracil base and the carboxylic acid group of 4-carboxypiperidine moiety binds to the P_0 and B_1 subsite, respectively, while in the other, the uracil base occupies the purine—preferring B_2 subsite but the 4-carboxypiperidine group points toward the solvent. Similar phenomena are also observed from the crystal structures of various cyclic alkylamino substituted 5′-modified uridine or thymidine nucleosides.[32] It shows that the anchoring point of all these inhibitors is the pyrimidine base which is bound at the B_1 subsite but the 5′-substituents are directed towards the solvent rather than the active site residues. However, in all the cases discussed above it has been observed that the cyclic nature of the substituent generates a hindrance to the efficient binding of these inhibitors to RNase A.[32,40] Therefore, we turned our attention to pyrimidine nucleosides modified with acyclic functional groups expected to have minimum conformational rigidity. Also, depending on the nature of 'X' in XCH_2COOH, these compounds are expected to have better binding through enhanced electrostatic interactions.

2. Results and discussion

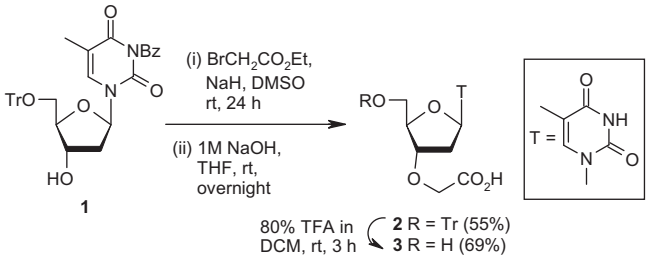
2.1. Synthesis of inhibitors

For the designing of inhibitors the nucleobase thymine was left unaltered for the recognition by the B_1 site. The $-XCH_2COOH$ (X=, O, N, S, SO_2) functionalities were attached to the sugar ring of the nucleoside. Thus, for the incorporation of the $-OCH_2COOH$ group at the 3′-position, N^3 of 5′-*O*-tritylthymidine was benzoylated following the literature procedure to synthesize compound **1**.[41,42] Compound **1** was reacted with bromoethylacetate in presence of NaH in dry DMSO for 24 h at room temperature followed by base hydrolysis to afford 3′-*O*-(carboxymethyl)-5′-*O*-tritylthymidine **2**[43] in 55% overall yield. Compound **2** was treated with 80% TFA in DCM for 3 h at room temperature to obtain compound **3** (T-3′OCH_2COOH)[44] in 69% yield (Scheme 1).

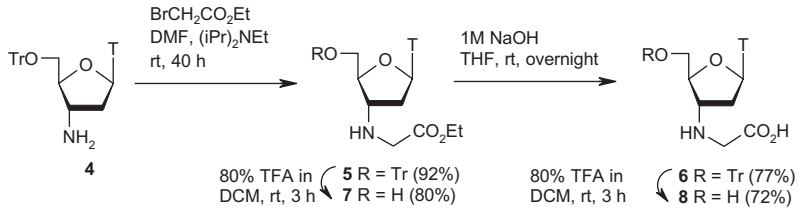
The synthesis of $-NHCH_2COOH$ was initiated by reacting aminothymidine **4**[45,46] with bromoethylacetate in presence of diisopropylethylamine in dry DMF at room temperature to afford 3′-*N*-(carboxymethyl)-5′-*O*-tritylthymidine **5** in 92% yield.[47] Bromoethylacetate (1 equiv) was added in portion (10 × 0.1 equiv) over a period of 40 h to get the monoalkylated product as major compound. Compound **5** was detritylated by 80% TFA in DCM for 3 h at room temperature to afford compound **7** (T-3′$NHCH_2COOEt$) in 80% yield. Compound **5** was hydrolyzed by aq 1 M NaOH solution to obtain compound **6** in 77% yield, which on acid treatment afforded compound **8** (T-3′$NHCH_2COOH$) in 72% yield (Scheme 2).

The preparation of thioglycolic acid modified thymidine required the reactions between the 'up'-mesylated nucleoside **9**[45] and the sodium salt of methyl 2-mercaptoacetate in DMF at 0 °C, which afforded compound **10** in 66% yield. Compound **10** on treatment with 80% TFA in DCM afforded the ester derivative **12** (T-3′SCH_2COOMe) in 79% yield. Another portion of compound **10** was hydrolyzed by 1 M NaOH in THF to afford compound **11** in 74% yield which was further converted to the free acid derivative **13** (T-3′SCH_2COOH) by 80% TFA in DCM. Compound **10** was treated with magnesium bis(monoperoxyphthalate)hexahydrate (MMPP) in dry MeOH at room temperature to obtain compound **14** in 70% yield which on treatment with 80% TFA in DCM afforded **15** (T-3′SO_2CH_2COOMe) in 75% yield. Again, compound **14** was converted to **16** (T-3′SO_2CH_2COOH) in 55% overall yield after consecutive hydrolysis by lithium hydroxide and 80% TFA (Scheme 3).

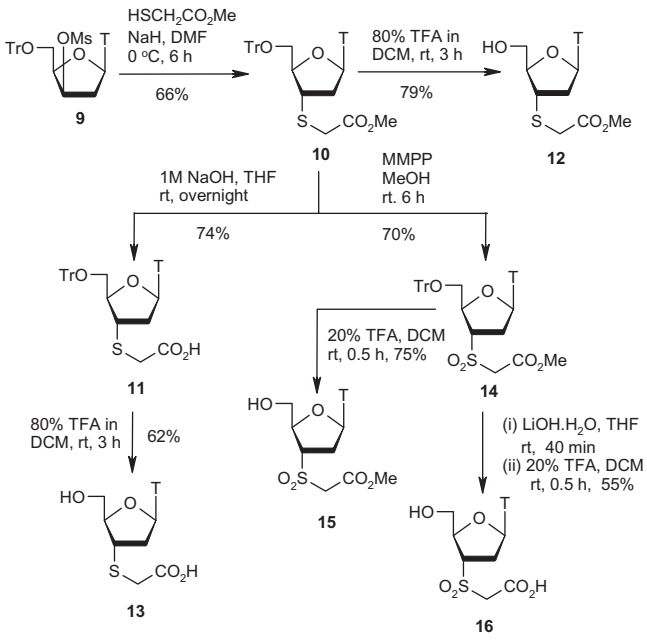
Since sulfone containing compounds showed better efficiency as inhibitors (see later) we decided to synthesize several related sulfonylated thymidines. For the incorporation of two thioglycolic acid



Scheme 1. Synthesis of T-3′OCH_2COOH **3**.



Scheme 2. Synthesis of T-3′NHCH$_2$COOH **8**.



Scheme 3. Synthesis of T-3′SCH$_2$COOH **13** and T-3′SO$_2$CH$_2$COOH **16**.

units, dithiothymidine **17**[48] was treated with ethylbromoacetate in DCM in presence of TEA.[49] Compound **18**, thus obtained was converted to its methyl ester analogue by a transesterification method[50] to afford **19** (T-3′,5′-bisSCH$_2$COOMe) in good yield.

Compound **19** was oxidized to the sulfone ester **20** (T-3′,5′-bisSO$_2$CH$_2$COOMe) and thereafter the latter compound was hydrolyzed under basic condition to afford the desired bis-acid **21** (T-3′,5′-bisSO$_2$CH$_2$COOH). The bis-ethylthioglycolate ester **18** was hydrolyzed by 1 M NaOH solution to obtain the corresponding bis-thioglycolic acid **22** (T-3′,5′-bisSCH$_2$COOH) in moderate yield (Scheme 4).

Ditosylate thymidine **23**[51] was treated with 2-mercaptoacetate using similar reaction condition[52] mentioned in Scheme 3 to obtain the desired bis-modified **24** [T-3′(up),5′-bisSCH$_2$COOMe] in high yield. Thereafter **24** was oxidized by MMPP to generate bis-sulfone ester **25** [T-3′(up),5′-bisSO$_2$CH$_2$COOMe], which on further hydrolysis afforded acid **26** [T-3′(up),5′-bisSO$_2$CH$_2$COOH]. Again, **24** was hydrolyzed under basic condition with 1 M NaOH solution to obtain the corresponding bis-thioglycolic acid derivative **27** [T-3′(up),5′-bisSCH$_2$COOH] in moderate yield (Scheme 5).

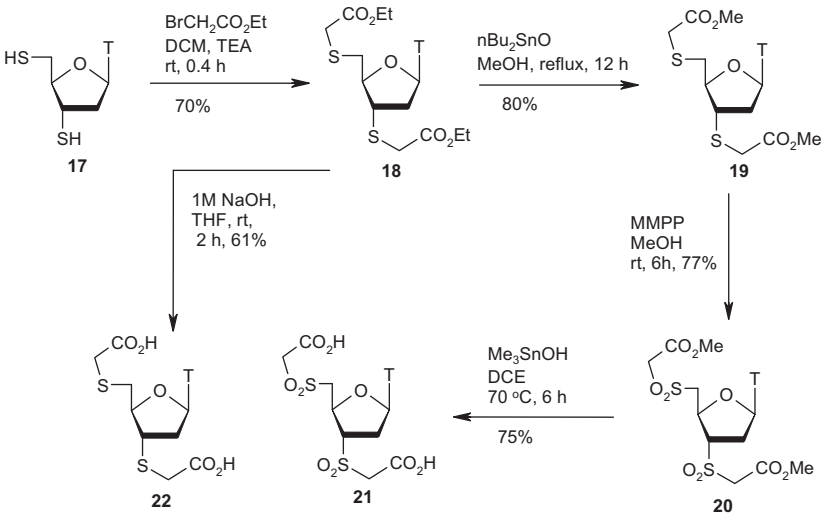
On the other hand, 5′-*O*-tosylthymidine **28**[33] was treated with the sodium salt of methyl 2-mercaptoacetate in dry DMF at room temperature to afford compound **29** (T-5′SCH$_2$COOMe)[53] in 76% yield. Ester **29** was oxidized first and then hydrolyzed under basic condition to obtain corresponding sulfone ester **30** (T-5′SO$_2$CH$_2$COOMe) and acid **31** (T-5′SO$_2$CH$_2$COOH) (Scheme 6). It should be noted that the yields of **21**, **26**, and **31** were too low under routine hydrolysis conditions which were later improved by applying trimethyltin hydroxide (TMTOH).[54]

2.1.1. Biophysical studies

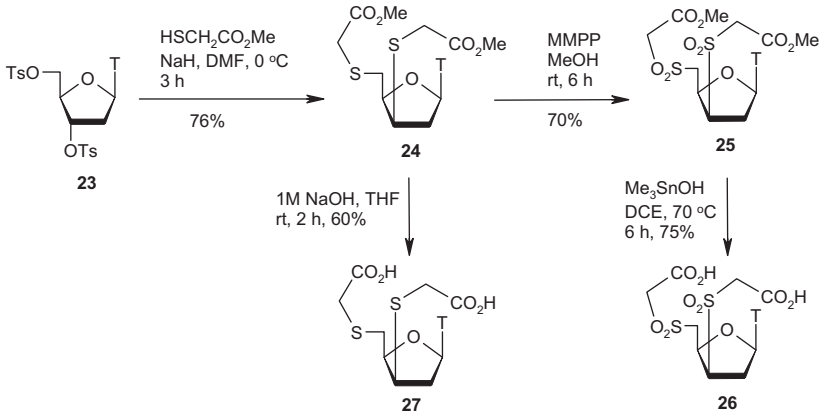
The agarose gel-based assay was performed to get a qualitative idea of the inhibitory activity of the synthesized compounds **3**, **7**, **8**, **12**, **13**, **15**, **16**, **19–22**, **24–27**, **30** and **31** against RNase A. In the agarose gel, the band in lane 1 showed maximum intensity due to the presence of only tRNA. Due to the degradation of tRNA by RNase A, the band in lane 2 was the least intense. Lanes 3, 4 and 5 of each gel contained tRNA and RNase A with increasing concentrations of synthesized compounds **3**, **7**, **8**, **12** and **13** [Fig. I in SI]. A significant difference in intensity between lanes 2 and 3 was observed for compounds **3**, (T-3′OCH$_2$COOH) **8** (T-3′NHCH$_2$COOH) and **13** (T-3′SCH$_2$COOH) and these differences gradually increased from lane 3–5. It was evident that, the intensity change was less prominent for the ester compounds, viz.; **7** (T-3′NHCH$_2$COOEt), **12** (T-3′SCH$_2$COOMe) **24** [T-3′(up),5′-bisSCH$_2$COOMe], and **19** (T-3′,5′-bisSCH$_2$COOMe). However, despite being esters, compounds **15** (T-3′SO$_2$CH$_2$COOMe), **20** (T-3′,5′-bisSO$_2$CH$_2$COOMe), **25** [T-3′(up),5′-bisSO$_2$CH$_2$COOMe], and **30** (T-5′SO$_2$CH$_2$COOMe) showed prominent intensity changes with increase in inhibitor concentration [Fig. I and II in SI]. Similar qualitative experiments have been performed for sulfone-acetic acid modified thymidines viz. compound **16** (T-3′SO$_2$CH$_2$COOH), **21** (T-3′,5′-bisSO$_2$CH$_2$COOH), **26** [T-3′(up),5′-bisSO$_2$CH$_2$COOH], **31** (T-5′SO$_2$CH$_2$COOH) and sulfur-acetic acid modified thymidines viz. **27** [T-3′(up),5′-bisSCH$_2$COOH] and **22** (T-3′,5′-bisSCH$_2$COOH) [Fig. III in SI]. In Figures 2 and 3, representative images of the agarose gel based electrophoresis assay have been given. From the gel images, it was evident that sulfone-acetic acids always show better inhibitory property than the other modified nucleosides mentioned above.

After confirming the possibility of inhibition by the agarose gel based assay, we performed the precipitation assay with compounds **3**, **7**, **8**, **12**, **13**, **15**, **16**, **19–22**, **24–27**, **30** and **31** against RNase A. The reduction of the ribonucleolytic activity of these compounds for RNase A (1.31 μM) at a fixed concentration (0.35 mM) was compared. The activity of RNase A was reduced to a greater extent by the acids in comparison to the esters, following a range of 13–35% for acids (Fig. 4) and 3–18% for esters (see Fig. IV in SI). Compound **3** (T-3′OCH$_2$COOH) exhibited 13% inhibition of ribonucleolytic activity compared to 19% by **8** (T-3′NHCH$_2$COOH). Thioglycolic acid **13** (T-3′SCH$_2$COOH) exhibited a 17% reduction in the ribonucleolytic activity. The 3′-substituted sulfone acid **16** (T-3′SO$_2$CH$_2$COOH) showed better inhibitory properties (25% reduction) compared to the 5′-analogue **31** (T-5′SO$_2$CH$_2$COOH) (22% reduction). The bis-thioglycolic acids **27** [T-3′(up),5′-bisSCH$_2$COOH] and **22** (T-3′,5′-bisSCH$_2$COOH) showed moderate potency and were able to reduce the ribonucleolytic activity by 16% and 18%, respectively, (Fig. 4).

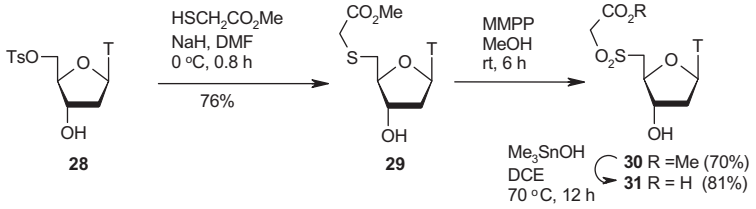
Except sulfone-modified esters tested in this assay, other esters like, **7** (T-3′NHCH$_2$COOEt), **12** (T-3′SCH$_2$CO$_2$Me), **24** [T-3′(up),5′-bisSCH$_2$COOMe] and **19** (T-3′,5′-bisSCH$_2$COOMe) exhibited very low inhibitory activity (7%, 3%, 5% and 6%, respectively). Reduction of ribonucleolytic property improved for sulfone-esters viz. **15** (T-3′SO$_2$CH$_2$COOMe), **25** [T-3′(up),5′-bisSO$_2$CH$_2$COOMe], **20** (T-3′,5′-bisSO$_2$CH$_2$COOMe), and **30** (T-5′SO$_2$CH$_2$COOMe) which showed



Scheme 4. Synthesis of T-3′,5′-bisSO_2CH_2COOH **21** and T-3′,5′-bisSCH_2COOH **22**.



Scheme 5. Synthesis of T-3′(up),5′-bisSO_2CH_2COOH **26** and T-3′(up),5′-bisSCH_2COOH **27**.



Scheme 6. Synthesis of T-5′SO_2CH_2COOH **31**.

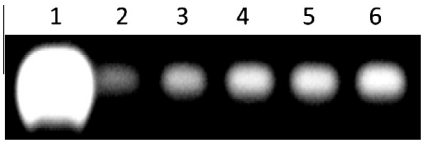


Figure 2. Agarose gel for the inhibition of RNase A (2.0 μM): (lane 1) tRNA; (lane 2) tRNA and RNase A; (lanes 3–6) tRNA, RNase A and **21** (T-3′,5′-bisSO_2CH_2COOH) (0.05, 0.10, 0.15 and 0.20 mM, respectively).

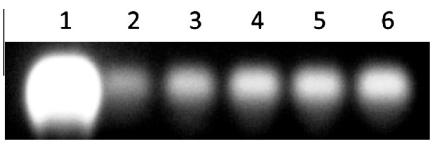


Figure 3. Agarose gel for the inhibition of RNase A (2.0 μM): (lane 1) tRNA; (lane 2) tRNA and RNase A; (lanes 3–6) tRNA, RNase A and **26** [T-3′(up),5′-bisSO_2CH_2COOH] (0.05, 0.10, 0.15 and 0.20 mM, respectively).

18%, 12%, 15%, 14% inhibition, respectively [Fig. IV in SI]. Among the ester and acids in this series, best results were shown by bis-sulfone acids **26** [T-3′(up),5′-bisSO_2CH_2COOH] and **21** (T-3′,5′-bisSO_2CH_2COOH) with almost equal potency (33% and 35%, respectively). From the two qualitative experimental observations, we could say that among the seventeen preliminarily compounds

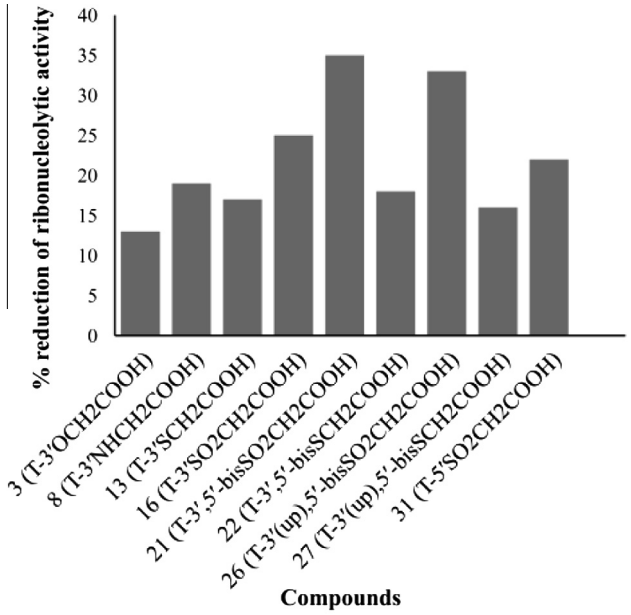


Figure 4. Reduction of ribonucleolytic activity of RNase A (1.31 μM) in the presence of acids (0.35 mM) by precipitation assay.

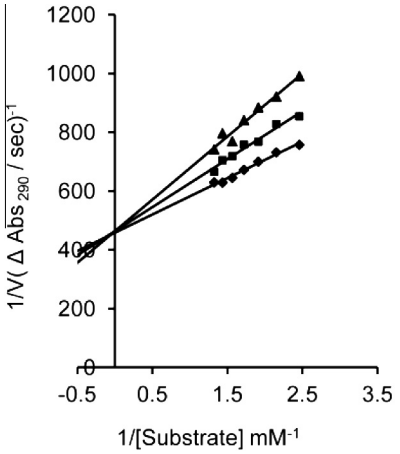


Figure 5. Lineweaver–Burk plots for inhibition of RNase A by **21** (T-3′,5′-bisSO_2CH_2COOH) of 19.0 (▲), 9.5 (■), 0.00 (♦)μM, 2′,3′-cCMP concentrations (0.77–0.42 mM) and RNase A concentration of 20.0 μM.

screened, acids have shown a better inhibitory activity compared to the corresponding esters.

To determine the mode of inhibition and inhibition constants (K_i) of compounds **3**, **7**, **8**, **12**, **13**, **15**, **16**, **20**–**22**, **25**–**27**, **30** and **31** steady state kinetic experiments were performed. The inhibition constants of potent inhibitors of RNase A, predicted from qualitative assays, were determined from the Lineweaver–Burk plots (SI). From the K_i values, it could be concluded that the thio and sulfone acetic acid modified thymidine compounds were more potent than compound **8** (T-3′$NHCH_2COOH$) and **3** (T-3′OCH_2COOH). The ester compounds **7** (T-3′$NHCH_2COOEt$) and **12** (T-3′SCH_2COOMe) showed weak inhibition property against the enzyme. However, sulfone-esters (**15**, **20**, **25**, and **30**) exhibited slightly better inhibition with moderate K_i values (Table 1) which was in agreement with the preliminary assays.

Finally, inhibition constants (K_i) for bis-sulfur and bis-sulfone compounds were calculated. A representative Lineweaver–Burk plot of compound **21** against RNase A has been depicted in Figure 5. The K_i values mentioned in Table 1 reflect that the fact that the thio-acids are much weaker inhibitors than sulfone and bis-sulfone acids. Bis-sulfone acid **21** is the most potent inhibitor in this series. This phenomenon can be explained as a result of greater H-bonding ability of this acid, designed with two sulfone groups. Thus, it is able to generate a larger H-bonding network in the proximity of the active site of the enzyme. In all the cases mentioned above, the nature of the plots indicated reversible competitive inhibition [Figs. V, VI and VII in SI].

Table 1
Inhibition constants (K_i) of inhibitors

Inhibitors (acids)	K_i (μM)	Inhibitors (esters)	K_i (μM)
3 ($T_{3'OCH2COOH}$)	267 ± 11	[a]	
8 ($T_{3'NHCH2COOH}$)	186 ± 9	**7** ($T_{3'NHCH2COOEt}$)	504 ± 20
13 ($T_{3'SCH2COOH}$)	169 ± 9	**12** ($T_{3'SCH2COOMe}$)	757 ± 22
16 ($T_{3'SO2CH2COOH}$)	88 ± 5	**15** ($T_{3'SO2CH2COOMe}$)	140 ± 4
21 ($T_{3',5'-bisSO2CH2COOH}$)	25 ± 2	**20** ($T_{3',5'-bisSO2CH2COOMe}$)	267 ± 5
22 ($T_{3',5'-bisSCH2COOH}$)	93 ± 4	**18** ($T_{3',5'-bisSCH2COOMe}$)	ND[b]
26 [$T_{3'(up),5'-bisSO2CH2COOH}$]	39 ± 2	**25** [$T_{3'(up),5'-bisSO2CH2COOMe}$]	242 ± 7
27 [$T_{3'(up),5'-bisSCH2COOH}$]	108 ± 5	**24** [$T_{3'(up),5'-bisSCH2COOMe}$]	ND[b]
31 ($T_{5'SO2CH2COOH}$)	95 ± 3	**30** ($T_{5'SO2CH2COOMe}$)	202 ± 3

ND due to very poor response in agarose gel-based assay.
[a] The ester $T_{3'OCH2COOEt}$ corresponding to the acid **3** not synthesized.
[b] Kinetic experiments not done (ND) due to very poor response in agarose gel-based assay.

2.1.2. Docking studies

We performed docking experiment to get probable binding conformations of the inhibitors with RNase A. In docking, the thymine base of 3′-modified acid compounds **3** (T-3′OCH_2COOH), **8** (T-3′$NHCH_2COOH$), **13** (T-3′SCH_2COOH) and **16** (T-3′SO_2CH_2COOH) has shown an improved interaction pattern with B_1 subsite which in turn, has been reflected in the observed inhibition constants also [Figs. VIII and XI in SI]. In the docked pose, for compound **3** (Fig. 6), the nucleobase occupies the active cleft of P_1 subsite consisting of His12, Lys41 and His119 (amino acid residues highlighted in cyan) which moves towards the B_1 subsite consisting of Val43, Asn44 and Thr45 amino acid residues (highlighted in green) as the substitution is changed from oxygen to SO_2 at the 3′-position of the inhibitors (Fig. 6). Quite remarkably, it is found that for the sulfone-acid **16** (T-3′SO_2CH_2COOH), there is appreciable inclination of the nucleobase towards the B_1 subsite (Fig. 6) which is likely due to the effect of the sulfone group present at the 3′-position which is able to generate more H-bonding interactions. Eventually, H-bonding interaction is observed for compound **16** with B_1 site (Fig. 6 and Tables II–IV in SI).

Binding of the acid groups of compound **3**, **8** and **13** are distinctly different. In the docked conformation, the acid group of compound **13** (T-3′SCH_2COOH) is within hydrogen bonding distance of the active site His12 and His119 residues while that of compound **8** (T-3′$NHCH_2COOH$) is near His119 residue only. On the other hand, the acid group of compound **3** (T-3′OCH_2COOH) is bound to the Gln11 residue and observed to be away from the active site His residues. From the docked structure it is noted that the 3′-hetero atom plays a vital role in binding. In compound **3**, the hydrogen bonding interaction of the 3′-oxygen atom with His119 hinders binding of the acid group with the His residues. For compound **8,** the 3′-NH is also hydrogen bonded with His119 and this interaction favors the binding of the acid group with His119 but not with His12. The 3′-sulfur atom of compound **13** interacts with Lys7 to facilitate the H-bonding interaction with His119 as well as His12 which is the probable reason for slightly higher potency compared to compound **3** and **8**. Compound **16** consisting of 'stronger' acid functionality, –SO_2CH_2COOH, that accommodates itself into the active site due to proper nucleobase recognition and additional electrostatic interactions. These parameters make it superior

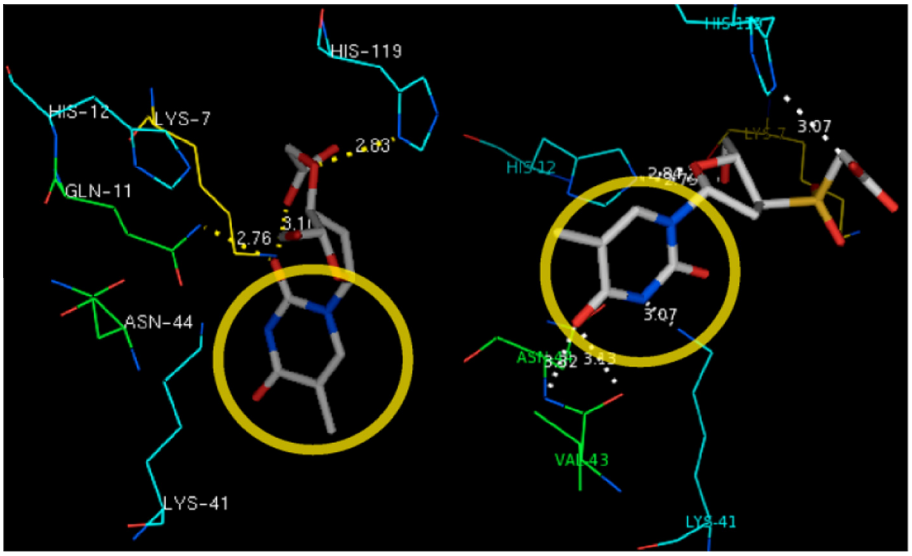


Figure 6. Docked poses of compound **3** (T-3′OCH$_2$COOH) (K_i = 267 ± 11 μM) and **16** (T-3′SO$_2$CH$_2$COOH) (K_i = 88 ± 5 μM) with RNase A (1FS3) where movement of nucleobase towards B_1 site (amino acid residues colored in green) is observed.

than the oxo-, amino- and thio-glycolic acid modified nucleoside inhibitors.

Ester compounds, **7** and **12** interact with RNase A through the thymine base but the 3′-ester substituent is away from the P_1 subsite. In spite of being esters, **15**, **20**, **25** and **30** perform as good inhibitors of RNase A due to additional interactions between the sulfone groups of the molecules and residues of the protein that lie within H-bonding distance [Figs IX and X in SI]. For 5′-modified compounds **30** and **31**, the 5′-substitutions appear to move away from the active site and interact weakly with the B_1 subsite [Fig. X in SI]. For **31**, the nucleobase was away from the B_1 subsite due to a possible H-bonding interaction with Lys41.

Finally, for **21** (T-3′,5′-bisSO$_2$CH$_2$COOH) and **26** [T-3′(up),5′-bisSO$_2$CH$_2$COOH] docking studies reveal H-bonding interactions with amino acid residues of the active site (P_1) and other subsites (P_0, P_2, B_2) [Tables III–IV in SI]. Docked conformations of both **21** (T-3′,5′-bisSO$_2$CH$_2$COOH) and **26** [T-3′(up),5′-bisSO$_2$CH$_2$COOH] depict favorable alignment for interactions with the enzyme active site. Here, due to proper recognition of two anchoring –SO$_2$CH$_2$COOH functionalities with different phosphate binding subsites (P_0 and P_1), **21** elicits the best inhibitory property among the aforementioned molecules. In the docked pose of **26** with RNase A, diminished H-bonding interactions with the P_1 subsite are noticeable due to a conformational change in the 3′-site of the inhibitor (Fig. 7).

This subtle change, affects the K_i value of **26**, making it less potent than **21**. The proximity of the –COOH groups with the effective side chain residues at the active site along with proper nucleobase recognition are the most probable reasons for better activity of these two sulfone-modified acids. Thus, docking results substantiate the biological assays to a large extent.

After docking studies, rationalization of the experimentally obtained inhibition constant (K_i) with docking parameters were checked with the aid of *PEARLS*.[55] During such analysis, the acids viz. **3**, **8**, **13**, **16**, **21**, **22**, **26**, **27**, **31** and ester **15**, which showed considerably low K_i values, were chosen and their protein–ligand merged PDB-format files were selected for total energy calculation of ligand–receptor interactions. The calculated protein–li-

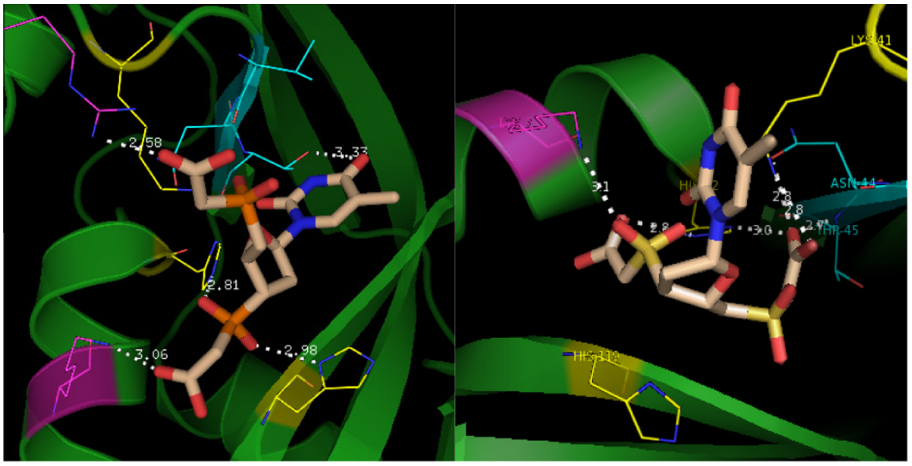


Figure 7. Docked poses of compound **21** (T-3′,5′-bisSO$_2$CH$_2$COOH) and **26** [T-3′(up),5′-bisSO$_2$CH$_2$COOH] with RNase A (1FS3) where cyan, yellow and magenta colored amino acid residues are of B_1, P_1 and other subsites respectively within H-bonding interaction.

Table 2
Theoretical total ligand–receptor interaction energy and experimentally obtained inhibition constants for correlation

Inhibitor/ligand	K_i (μM)	Total ligand–receptor interaction energy (kcal/mol)
3 (T-3′OCH_2COOH)	267	−5.65
8 (T-3′-$NHCH_2COOH$)	186	−6.61
13 (T-3′SCH_2COOH)	169	−5.84
15 (T-3′SO_2CH_2COOMe)	140	−8.48
16 (T-3′SO_2CH_2COOH)	88	−8.23
21 (T-3′,5′-bisSO_2CH_2COOH)	25	−9.25
22 (T-3′,5′-bisSCH_2COOH)	93	−6.13
26 [T-3′(up),5′-bisSO_2CH_2COOH]	39	−9.40
27 [T-3′(up),5′-bisSCH_2COOH]	108	−6.10
31 (T-5′SO_2CH_2COOH)	95	−7.73

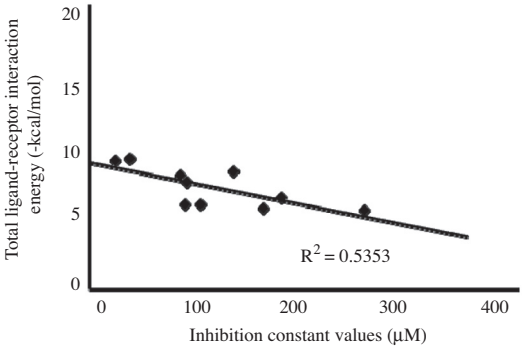


Figure 8. A correlation graph of ligand–receptor interaction and inhibition constant values.

gand (here, RNase A and inhibitors of RNase A) interaction values were obtained from the automated program *PEARLS* and the data tabulated (Table 2). A correlation graph has been plotted with the help of obtained data and corresponding inhibition constants (Fig. 8). The linear nature of the curve suggests a distinct correlation between theoretically and experimentally obtained data sets.

3. Conclusion

Since nucleosides functionalized with acidic groups are known to act as efficient inhibitors of P_1 subsite of RNase A,[32] we designed a family of 3′-functionalized thymidines carrying XCH_2COOH (X = O, NH, S, SO_2) because in this series it would be possible to manipulate the overall acidity of this new class of nucleic 'acids' by changing X from oxygen to the SO_2 group. As expected, 3′-deoxy- and 5′-deoxy-(3′-oxy-, amino- and thio) acetic acid modified pyrimidine nucleosides were identified as moderate inhibitors of RNase A. The sulfone acetic acid modified thymidine compounds, expected to be the most acidic nucleosides in this series were found to be the more potent inhibitors of RNase A. A bis-modified thymidine armed with two $-SO_2CH_2COOH$ groups at the 3′ and 5′ sites was found to be the most effective inhibitors in the series with a K_i value 25 ± 2 μM. It was observed in the docking studies that the $-CH_2COOH$ group of these compounds was within H-bonding distance of the active site (P_1) of the enzyme. The acyclic nature of XCH_2COOH also helped the carboxylic function to easily reach the target site. Moreover, in the case of $-SO_2CH_2COOH$ functionalized thymidines, additional binding patterns of the oxygen atom(s) of the SO_2 group, makes these molecules more effective inhibitors. All these phenomena lead to suitable recognition of inhibitors, thereby enhanced their potency as initially presumed. Extensive docking studies corroborated experimentally obtained data quite well which in turn, strengthen our approach towards the design and synthesis of RNase A inhibitors.

4. Materials and methods

All reagents were commercially purchased. Column chromatographic separations were done using silica gel (60–120 and 230–400 mesh). Solvents were dried and distilled following standard procedures. TLC was carried out on precoated 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 in MeOH, 5% vanillin in MeOH and 5% ninhydrin in *n*-butanol solutions. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on Bruker NMR spectrometer unless stated otherwise. All ^1H NMR in D_2O were recorded using HPLC grade acetonitrile as internal standard. All ^{13}C NMR spectra in D_2O were recorded using DMSO-d_6 or HPLC grade acetonitrile as internal standard. Chemical shifts are reported in parts per million (ppm, δ scale). DEPT experiments have been carried out to identify the methylene carbons. Mass spectroscopy data were obtained from Xevo G2QTof mass spectrometer in either ESI^+ or ESI^- mode. Melting points were determined in open-end capillary tubes and are uncorrected. Bovine pancreatic RNase A, yeast tRNA, 2′, 3′-cCMP, 3′-CMP and human serum albumin (HSA) were purchased commercially. UV–Vis measurements were made using a UV–Vis spectrophotometer (Model Lambda 25). Concentrations of the solutions were estimated spectrophotometrically using the following data: $\varepsilon_{278.5} = 9800\ M^{-1}\ cm^{-1}$ (RNase A)[56] and $\varepsilon_{268} = 8500\ M^{-1}\ cm^{-1}$ (2′, 3′-cCMP).[57]

Purity of two most active compounds, **21** and **26** were analyzed for purity on Chiralpak OJ-H equipped reverse phase column (0.46 × 15 cm) having flow rate of 1.0 mL/min, λ = 254 nm. The mobile phase was a mixture of isopropanol and hexane. Applied gradient was 40–60% isopropanol over 15 min using Chiralpak OJ-H column. These compounds were more than 96% pure.

4.1. Chemistry

4.1.1. 3′-Deoxy-3′-*O*-[(carboxymethyl)oxo]-5′-*O*-tritylthymidine 2

Compound **2** was synthesized according to the reported procedure[43] with the following modification. Compound **1** (0.43 g, 0.74 mmol) in dimethylsulfoxide (DMSO) (5 mL) was added to the mixture of NaH (60% in dispersed in mineral oil, 0.036 g, 1 mmol) in DMSO (5 mL) at room temperature under N_2 atmosphere which was stirred for 1 h. Bromoethylacetate (0.14 mL, 1.10 mmol) was added to the mixture. After 12 h, another 0.14 mL (1.10 mmol) bromoethylacetate was added to the reaction mixture and the mixture was stirred for 12 h. Satd aq NH_4Cl solution was added to the reaction mixture and was partitioned and washed with ethylacetate (EtOAc) (2 × 50 mL). Combined organic layers were washed with brine, separated, dried over anhyd Na_2SO_4 and filtered. The filtrate was evaporated to dryness under reduced pressure. The crude residue was dissolved in a mixture of aq NaOH solution (1 M, 7.5 mL) and tetrahydrofuran (THF) (10 mL) and the solution was stirred overnight. Volatile matters were removed under reduced pressure and the residue was neutralized carefully with 1 M HCl. The solution was washed with dichloromethane (3 × 50 mL). Combined organic layers were washed with brine, separated, dried over anhyd Na_2SO_4 and filtered. The filtrate was concentrated and the residue thus obtained, was purified over a silica gel column to afford compound **2** (0.22 g, 55% in two steps). [Eluent: 0–10% of MeOH in $CHCl_3$]. Hygroscopic solid; ^1H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.15–1.18 ($[CH_3CH_2]_3N$), 1.43 (s, 3H), 2.24–2.31 (m, 1H), 2.92–2.98 ($[CH_3CH_2]_3N$), 3.30–3.37 (m, 2H), 3.93 (br s, 1H), 4.45–4.50 (m, 3H), 6.30–6.33 (m,

1H), 7.16–7.25 (m, 9H), 7.33–7.35 (m, 6H), 7.48 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 8.4 ($[CH_3CH_2]_3N$), 12.6, 40.9 (CH_2), 43.9 (CH_2), 45.1 ($[CH_3CH_2]_3N$), 63.8 (CH_2), 71.6, 85.4, 85.8, 87.2 (C), 109.9 (C), 127.2, 127.9, 128.6, 133.9, 143.4 (C), 150.8 (C), 163.3 (C), 172.6 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+H)^+$ $C_{31}H_{31}N_2O_7$: 543.2131, found: 543.2139.

4.1.2. 3′-Deoxy-3′-*O*-[(carboxymethyl)oxo]thymidine (T-3′OCH$_2$COOH) 3

Compound **2** (0.25 g, 0.46 mmol) was stirred with trifluoroacetic acid (TFA) in DCM (80%, 5 mL) at room temperature. After 3 h, the volatile matters were evaporated to dryness under reduced pressure and residual liquid was co-evaporated with toluene (2 × 5 mL). The residue, thus obtained was dissolved in MeOH (2–3 mL), loaded onto the silica gel column, and purified to obtain compound **3** (0.96 g, 69%) [Eluent: 5–20% of MeOH in $CHCl_3$]. Hygroscopic solid; ^{1}H NMR (D_2O, 25 °C): δ = 1.92 (s, 3H), 2.38–2.40 (m, 2H), 3.74–3.86 (m, 2H), 4.02 (s, 1H), 4.45–4.51 (m, 3H), 6.30 (t, *J* = 6.4 Hz, 1H), 7.70 ppm (s, 1H); ^{13}C NMR (D_2O, 25 °C): δ = 13.9, 40.4 (CH_2), 45.6 (CH_2), 62.6 (CH_2), 71.8, 87.3, 88.1, 111.7 (C), 137.2, 152.8 (C), 166.1 (C), 174.8 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+Na)^+$ $C_{12}H_{16}N_2O_7Na$: 323.0856, found: 323.0861.

4.1.3. 3′-Deoxy-3′-*N*-[(ethoxycarbonyl)methylamino]-5′-*O*-tritylthymidine 5

To a suspension of compound **4** (1.22 g, 2.52 mmol) in DMF (30 mL), diisopropylethylamine (1.32 mL, 7.56 mmol) was added under N_2 atmosphere at room temperature. Bromoethylacetate was added in portions (0.1 × 10 equiv) to the reaction mixture over a period of 40 h.[47] Satd aq $NaHCO_3$ solution was added to the reaction mixture and the aq solution was washed with EtOAc (2 × 50 mL). Combined organic layers were washed with brine, dried over anhyd Na_2SO_4 and filtered. The filtrate was removed under reduced pressure and the residue was purified over a silica gel column to afford compound **5** (1.32 g, 92%) [Eluent: 50–60% of EtOAc in pet ether]. White solid; mp 121–124 °C; ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.24 (t, *J* = 7.2 Hz, 3H), 1.50 (s, 3H), 2.26–2.31 (m, 2H), 3.30–3.34 (m, 3H), 3.49–3.52 (m, 1H), 3.56–3.61 (m, 1H), 3.91–3.92 (m, 1H), 4.13–4.19 (m, 1H), 6.27 (t, *J* = 6.0 Hz, 1H), 7.24–7.33 (m, 9H), 7.42–7.44 (m, 6H), 7.59 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 11.9, 14.1, 38.9 (CH_2), 48.9 (CH_2), 57.5, 61.0 (CH_2), 63.6 (CH_2), 84.6, 84.8, 87.3(C), 110.8 (C), 127.3, 127.9, 128.6, 135.5, 143.3 (C), 150.3 (C), 163.9 (C), 171.9 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+H)^+$ $C_{33}H_{36}N_3O_6$: 570.2604, found: 570.2615.

4.1.4. 3′-Deoxy-3′-*N*-[(carboxymethyl)amino]-5′-*O*-tritylthymidine 6

Compound **5** (0.76 g, 1.33 mmol) was converted to compound **6** (0.53 g, 77%) following the method described for compound **2** [Eluent: 2–10% of MeOH in $CHCl_3$]. White solid; mp 162–165 °C; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.45 (s, 3H), 2.24 (br s, 2H), 3.18–3.27 (m, 4H), 3.56–3.57 (m, 1H), 3.94 (br s, 1H), 6.20 (t, *J* = 6.0 Hz, 1H), 7.25–7.28 (m, 3H), 7.31–7.35 (m, 6H), 7.39–7.50 (m, 6H), 7.50 (s, 1H), 11.35 ppm (br s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.2, 36.9 (CH_2), 49.7 (CH_2), 58.0, 64.8 (CH_2), 83.1, 84.2, 86.7(C), 109.9 (C), 127.5, 128.4, 128.7, 136.1, 143.9 (C), 150.8 (C), 164.1 (C), 173.0 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+Na)^+$ $C_{31}H_{31}N_3O_6Na$: 564.2111, found: 564.2118.

4.1.5. 3′-Deoxy-3′-*N*-[(ethoxycarbonyl)methylamino]thymidine (T-3′NHCH$_2$COOEt) 7

Compound **5** (0.30 g, 0.53 mmol) was converted to compound **7** (0.14 g, 80%) following the method described for compound **3** [Eluent: 0–3% of MeOH in $CHCl_3$]. Hygroscopic solid; ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.27 (t, *J* = 7.2 Hz, 3H), 1.89 (s, 3H), 2.18–2.25 (m, 1H), 2.30–2.37 (m, 1H), 3.39–3.51 (m, 3H), 3.81–3.95 (m, 3H), 4.20 (q, *J* = 14.4 Hz, 2H), 6.12–6.15 (m, 1H), 7.46 (s, 1H), 9.12 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 12.5, 14.1, 38.5 (CH_2), 49.0 (CH_2), 57.2, 61.2 (CH_2), 62.4 (CH_2), 85.2, 86.0, 110.7 (C), 136.5, 150.3 (C), 163.9 (C), 172.3 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+H)^+$ $C_{14}H_{22}N_3O_6$: 328.1508, found: 328.1517.

4.1.6. 3′-Deoxy-3′-*N*-[(carboxymethyl)amino)]thymidine (T-3′NHCH$_2$COOH) 8

Compound **6** (0.40 g, 0.74 mmol) was converted to compound **8** (0.16 g, 72%) following the method described for compound **3** [Eluent: 5–20% of MeOH in $CHCl_3$]. Hygroscopic solid; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.77 (s, 3H), 2.12–2.17 (m, 2H), 3.25 (s, 2H), 3.41–3.42 (m, 1H), 3.54–3.65 (m, 2H), 3.80–3.81 (m, 1H), 6.15 (t, *J* = 6.4 Hz, 1H), 7.73 (s, 1H), 11.28 ppm (br s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.7, 36.9 (CH_2), 49.1 (CH_2), 57.7, 62.0 (CH_2), 84.1, 84.6, 109.7 (C), 136.6, 150.9 (C), 164.2 (C), 174.5 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+H)^+$ $C_{12}H_{18}N_3O_6$: 300.1195, found: 300.1190.

4.1.7. 3′-Deoxy-3′-*S*-[(methoxycarbonyl)methylthio]-5′-*O*-tritylthymidine 10

To a suspension of NaH (60% in dispersed in mineral oil, 0.19 g, 8 mmol) in DMF (10 mL), methyl 2-mercaptoacetate (0.64 mL, 7 mmol) was added dropwisely at 0 °C under argon. The suspension was allowed to warm at room temperature while the solution turned yellow. The reaction mixture was cooled to 0 °C and compound **9**[45] (1.12 g, 2 mmol) in DMF (5 mL) was added to it. After 6 h, Satd aq NH_4Cl solution was added to the reaction mixture and was partitioned and washed with EtOAc (2 × 50 mL). Combined organic layers were washed with brine, separated, dried over anhyd Na_2SO_4 and filtered. The filtrate was removed under reduced pressure and the residue was purified over a silica gel column to afford compound **10** (0.74 g, 66%) [Eluent: 50–60% of EtOAc in pet ether]. White solid; mp 60–63 °C; ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.44 (s, 3H), 2.41–2.49 (m, 1H), 2.57–2.63 (m, 1H), 3.17 (s, 2H), 3.34–3.42 (m, 1H), 3.59–3.68 (m, 4H), 3.84–3.88 (m, 1H), 3.90–3.98 (m, 1H), 6.19–6.21 (m, 1H), 7.27–7.34 (m, 9H), 7.38–7.43 (m, 6H), 7.70 (s, 1H), 8.25 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 11.9, 32.9 (CH_2), 40.1 (CH_2), 41.3, 52.6, 62.2 (CH_2), 84.6, 84.9, 87.2(C), 110.9 (C), 127.4, 127.9, 128.8, 135.4, 143.2 (C), 150.2 (C), 163.8 (C), 170.2 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+Na)^+$ $C_{32}H_{32}N_2O_6SNa$: 595.1879, found: 595.1887.

4.1.8. 3′-Deoxy-3′-*S*-[(carboxymethyl)thio]-5′-*O*-tritylthymidine 11

Compound **10** (0.30 g, 0.52 mmol) was converted to compound **11** (0.22 g, 74%) following the method described for compound **2** [Eluent: 0–8% of MeOH in $CHCl_3$]. White solid; mp 185 °C (decompose); ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.43 (s, 3H), 2.47–2.54 (m, 1H), 2.60–2.66 (m, 1H), 3.19 (s, 2H), 3.37–3.41 (m, 1H), 3.59–3.62 (m, 1H), 3.81–3.85 (m, 1H), 3.95–3.97 (m, 1H), 6.12–6.14 (m, 1H), 7.24–7.33 (m, 9H), 7.42–7.43 (m, 6H), 7.75 (s, 1H), 9.58 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 11.9, 33.3 (CH_2), 40.4 (CH_2), 41.6, 62.2 (CH_2), 85.0, 85.1, 87.4 (C), 110.7 (C), 127.5, 128.1, 128.7, 136.2, 143.2 (C), 150.4 (C), 165.0 (C), 174.2 ppm (C); HRMS (ESI^+), *m*/*z* calcd for $(M+H)^+$ $C_{31}H_{31}N_2O_6S$: 559.1903, found: 559.1911.

4.1.9. 3′-Deoxy-3′-*S*-[(methoxycarbonyl)methylthio]thymidine (T-3′SCH$_2$COOMe) 12

Compound **10** (0.46 g, 0.8 mmol) was converted to compound **12** (0.21 g, 79%) following the method described for compound **3** [Eluent: 0–4% of MeOH in $CHCl_3$]. Hygroscopic solid; ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.86 (s, 3H), 2.36–2.43 (m, 1H), 2.54–2.56

(m, 1H), 3.34 (s, 2H), 3.59–3.65 (m, 1H), 3.73 (s, 3H), 3.78–3.89 (m, 2H), 3.97–4.00 (m, 1H), 6.09 (br s, 1H), 7.65 (s, 1H), 9.69 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 12.4, 33.2 (CH_2), 39.5 (CH_2), 41.1, 52.8, 60.9 (CH_2), 85.3, 85.9, 110.6 (C), 136.6, 150.5 (C), 164.3 (C), 170.9 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+H)^+$ $C_{13}H_{19}N_2O_6S$: 331.0964, found: 331.0973.

4.1.10. 3′-Deoxy-3′-*S*-[(carboxymethyl)thio]thymidine (T-3′SCH$_2$COOH) 13

Compound **11** (0.22 g, 0.39 mmol) was converted to compound **13** (0.08 g, 62%) following the method described for compound **3** [Eluent: 5–20% of MeOH in $CHCl_3$]. Hygroscopic solid; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.76 (s, 3H), 2.22–2.29 (m, 1H), 2.34–2.41 (m, 1H), 3.27 (s, 2H), 3.51–3.63 (m, 1H), 3.67–3.70 (m, 1H), 3.78–3.80 (m, 1H), 6.04 (t, *J* = 5.6 Hz, 1H), 7.84 ppm (s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.6, 35.5 (CH_2), 38.9 (CH_2), 41.6, 61.1 (CH_2), 84.0, 86.0, 109.4 (C), 136.7, 150.8 (C), 164.2 (C), 173.9 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+Na)^+$ $C_{12}H_{16}N_2O_6S$: 339.0627, found: 339.0626.

4.1.11. 3′-Deoxy-3′-*S*-[(methoxycarbonyl)methylsulfonyl]-5′-*O*-tritylthymidine 14

MMPP (0.65 g, 1.32 mmol) was added to a solution of **10** (0.25 g, 0.44 mmol) in anhyd MeOH and the mixture was stirred at room temperature and N_2 atmosphere. After 8 h, this reaction mixture was concentrated, treated with Satd aq $NaHCO_3$ and the solution was washed with EtOAc (2 × 25 mL). Organic layers were separated, dried over anhyd Na_2SO_4, filtered and the filtrate was concentrated under reduced pressure. The residue thus obtained was purified over silica gel column to afford compound **14** (0.2 g, 76%) [Eluent: 70% of EtOAc in pet ether].White solid; mp 108–112 °C; ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.47 (s, 3H), 2.55–2.59 (m, 1H), 3.00–3.06 (m, 1H), 3.45–3.48 (m, 1H), 3.62–3.65 (m, 1H), 3.76 (s, 3H), 3.96 (d, *J* = 14.8 Hz, 1H), 4.08 (d, *J* = 15.2 Hz, 1H), 4.63–4.66(m, 2H), 6.25–6.28 (m, 1H), 7.25–7.47(m, 15H), 7.56 (s, 1H). 9.29 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 12.0, 34.1 (CH_2), 53.7, 56.9 (CH_2), 60.8, 64.0 (CH_2), 77.6, 85.2, 87.8 (C), 111.7 (C), 127.7, 128.3, 128.8, 135.3, 143.3 (C), 150.3 (C), 163.3 (C), 164.0 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+Na)^+$ $C_{32}H_{32}N_2O_8S$: 627.1777, found: 627.1770.

4.1.12. 3′-Deoxy-3′-*S*-[(methoxycarbonyl) methylsulfonyl]thymidine (T-3′SO$_2$CH$_2$COOMe) 15

Compound **14** (0.1 g, 0.17 mmol) was stirred with TFA in DCM (20%, 15 mL) at room temperature. After 0.5 h, the volatile matters were evaporated to dryness under reduced pressure and residual liquid was co-evaporated with the mixture of toluene and carbon tetrachloride. The residue was triturated well with chilled diethylether (Et_2O) to obtain solid compound **15** (0.059 g, 93%). White solid; mp 195–198 °C; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.78 (s, 3H), 2.38–2.46 (m, 1H), 2.69–2.74 (m, 1H), 3.58–3.61 (m, 1H), 3.73–3.76 (m, 4H), 4.26–4.28 (m, 1H), 4.43(br s, 1H), 4.62–4.66 (m, 2H), 6.11–6.15 (m, 1H), 7.74 (s, 1H), 11.36 ppm (s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.6, 32.1 (CH_2), 53.3, 56.2 (CH_2), 61.9, 62.7 (CH_2), 78.2, 84.3, 110.1 (C), 136.3, 150.8 (C), 163.8 (C), 164.1 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+H)^+$ $C_{13}H_{18}N_2O_8S$: 363.0862, found: 363.0867.

4.1.13. 3′-Deoxy-3′-*S*-[(carboxymethyl)sulfonyl]thymidine (T-3′SO$_2$CH$_2$COOH) 16

LiOH·H_2O (0.03 g, 0.68 mmol) and water (4 mL) were added to a solution of compound **14** (0.1 g, 0.17 mmol) in THF (16 mL). The reaction mixture was stirred for 1 h and volatile matters were removed under reduced pressure. Solid $NaHSO_4$·H_2O (0.1 g) was added in portions to the residue and the mixture was stirred until the pH reached 7. The residue was diluted with water (10 mL) and the solution thus obtained, was washed with EtOAc (3 × 15 mL). Combined organic layers were separated, dried over anhyd Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure. Crude solid thus obtained, was converted to compound **16** (0.032 g, 55%) following the method described for compound **15**. White solid; mp 210–215 °C; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.76 (s, 3H), 2.34–2.42 (m, 1H), 2.67–2.72 (m, 1H), 3.55–3.58 (m, 1H), 3.71–3.74 (m, 1H), 4.25–4.28 (m, 1H), 4.39–4.49 (m, 3H), 6.08–6.12 (m, 1H), 7.72 (s, 1H), 11.33 ppm (s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.6, 32.1 (CH_2), 56.7 (CH_2), 61.7, 62.8 (CH_2), 78.2, 84.3, 110.1 (C), 136.3, 150.8 (C), 164.1 (C), 164.6 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+H)^+$ $C_{12}H_{16}N_2O_8S$: 349.0706, found: 349.0719.

4.1.14. 3′,5′-Dideoxy-3′,5′-bis-*S*-[(ethoxycarbonyl) methylthio]thymidine 18

To a solution of compound **17**[48] (0.4 g, 1.45 mmol) in dry DCM (5 mL), TEA (0.48 mL, 3.5 mmol) was added at 0 °C. To the resulting mixture, ethylbromoacetate (0.41 mL, 3.5 mmol) was added slowly. The reaction mixture was quenched with cold water after 0.4 h and the aq layer was washed with DCM (2 × 20 mL). Organic layers were separated, dried over anhyd $CaCl_2$, filtered and the filtrate was concentrated under reduced pressure. Crude product thus obtained, was purified over column to afford compound **18** (0.39 g, 70%) [Eluent: 40–60% of EtOAc in pet ether]. Transparent yellow gum; ^{1}H NMR (200 MHz, $CDCl_3$, 25 °C, TMS): δ = 1.25–1.33 (m, 6H), 1.95 (s, 3H), 2.45–2.52 (m, 2H), 3.00–3.24 (m, 2H), 3.25–3.42 (m, 4H), 3.46–3.58 (m, 1H), 4.01–4.09 (m, 1H), 4.14–4.26 (m, 4H) 6.15–6.21 (m, 1H), 7.43 (s, 1H), 9.26 ppm (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$, 25 °C, TMS): δ = 12.7, 14.3, 33.6 (CH_2), 34.6 (CH_2), 34.7 (CH_2), 40.0 (CH_2), 44.9, 61.7(CH_2), 61.9(CH_2), 84.3, 84.6, 111.5 (C), 135.6, 150.4(C), 163.9 (C), 170.1 (C), 170.3 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+Na)^+$ $C_{18}H_{26}N_2O_7S_2Na$: 469.1079, found: 469.1097.

4.1.15. 3′,5′-Dideoxy-3′,5′-bis-*S*-[(methoxycarbonyl)methylthio]thymidine (T-3′,5′-bisSCH$_2$COOMe) 19

A mixture of compound **18** (0.13 g, 0.29 mmol) and dibutyltin oxide (0.014 g, 0.058 mmol) in MeOH was heated under reflux for 12 h. After completion of reaction (TLC), MeOH was removed under reduced pressure to obtain a crude mass which was purified over silica gel column to obtain **19** (0.1 g, 80%) [Eluent: 50–80% of EtOAc in pet ether]. Colorless gum; ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.92 (s, 3H), 2.43–2.47 (m, 2H), 3.02 (dd, *J* = 5.2, 14.4 Hz, 1H), 3.15 (dd, *J* = 3.6, 14.4 Hz, 1H), 3.28–3.38 (m, 4H), 3.45–3.52 (m, 1H), 3.71 (s, 3H), 3.73 (s, 3H), 3.99–4.04 (m, 1H), 6.13–6.16 (m, 1H), 7.38 (s, 1H), 9.47 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 12.7, 33.3 (CH_2), 34.3 (CH_2), 34.7 (CH_2), 39.9 (CH_2), 45.0, 52.7, 52.9, 84.3, 84.7, 111.5 (C), 135.6, 150.5 (C), 164.1 (C), 170.6 (C), 170.7 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+H)^+$ $C_{16}H_{23}N_2O_7S_2$: 419.0947, found: 419.0939.

4.1.16. 3′,5′-Dideoxy-3′,5′-bis-*S*-[(methoxycarbonyl) methylsulfonyl]cthymidine (T-3′,5′-bisSO$_2$CH$_2$COOMe) 20

Compound **19** (0.6 g, 1.24 mmol) was converted to compound **20** (0.53 g, 77%) following the method described for compound **14**. [Eluent: 6% of MeOH in dichloromethane].White solid; mp 76–80 °C; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.78 (s, 3H), 2.59–2.67 (m, 1H), 2.72–2.78 (m, 1H), 3.64–3.73 (m, 4H), 3.77 (s, 3H), 4.07–4.13 (m, 1H), 4.41 (s, 2H), 4.43–4.48 (m, 1H), 4.61–4.71 (m, 2H), 4.75–4.78 (m, 1H), 6.08–6.11 (m, 1H), 7.56 (s, 1H), 11.39 ppm (s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.6, 31.2 (CH_2), 53.3, 53.6, 57.0 (CH_2), 57.7 (CH_2), 58.7 (CH_2), 64.4, 72.3, 86.4, 110.5 (C), 137.3, 150.9 (C), 163.7 (C), 164.1 (C), 164.3 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+H)^+$ $C_{16}H_{23}N_2O_{11}S_2$: 483.0743, found: 483.0757.

4.1.17. 3′,5′-Dideoxy-3′,5′-bis-*S*-[(carboxymethyl)sulfonyl] thymidine (T-3′,5′-bisSO_2CH_2COOH) 21

Compound **20** (0.05 g, 0.11 mmol) and trimethyltin hydroxide (TMTOH) (0.098 g, 0.55 mmol) dissolved in 1,2-dichloroethane (DCE) was heated at 70 °C for 6 h. After completion of reaction, volatile matters were evaporated under reduced pressure and the crude mass thus obtained, was purified over silica gel column to obtain compound **21** (0.035 g, 75%) [Eluent: 30–80% of MeOH in chloroform]. Yellowish white hygroscopic solid; ^{1}H NMR (D_2O, 25 °C, water suppressed): δ = 1.91 (s, 3H), 2.73–2.81 (m, 1H), 2.92–2.99 (m, 1H), 3.86–3.89 (m, 1H), 4.09–4.23 (m, 5H), 4.56–4.62 (m, 1H), 4.99–5.04 (m, 1H), 6.16–6.19 (m, 1H), 7.54 ppm (s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.6, 31.3 (CH_2), 57.1 (CH_2), 57.9 (CH_2), 58.7 (CH_2), 64.5, 72.3, 86.4, 110.6 (C), 137.3, 150.8 (C), 163.1 (C), 163.6 (C), 164.3 ppm (C); HRMS (ESI$^-$), *m*/*z* calcd for $(M-H)^-$ $C_{14}H_{17}N_2O_{11}S_2$: 453.0274, found: 453.0252.

4.1.18. 3′,5′-Dideoxy-3′,5′-bis-*S*-[(carboxymethyl)thio] thymidine (T-3′,5′-bisSCH_2COOH) 22

Compound **18** (0.091 g, 0.22 mmol) was dissolved in a mixture of aq NaOH solution (1 M, 2.5 mL) and THF (5 mL) and this solution was stirred at room temperature for 2 h. Volatile matters were removed under reduced pressure and the solution was neutralized with 1 M HCl solution. The resulting solution was evaporated to dryness under reduced pressure and the crude mass thus obtained, was purified over silica gel column to obtain compound **22** (0.048 g, 61%) [Eluent: 5–80% of MeOH in chloroform]. Hygroscopic yellowish white solid; ^{1}H NMR (D_2O, 25 °C): δ = 1.90 (s, 3H), 2.49–2.55 (m, 1H), 2.61–2.66 (m, 1H), 2.95 (dd, *J* = 6.4, 14.4 Hz, 1H), 3.10 (dd, *J* = 3.6, 14.4 Hz, 1H), 3.30–3.35 (m, 4H), 3.47–3.54 (m, 1H), 4.10–4.15 (m, 1H), 6.20–6.23 (m, 1H), 7.59 ppm (s, 1H); ^{13}C NMR (D_2O, 25 °C): δ = 12.2, 35.0 (CH_2), 36.3 (CH_2), 37.4 (CH_2), 38.5 (CH_2), 45.3, 84.6, 85.4, 112.2 (C), 138.2, 152.3 (C), 167.1 (C), 177.3 (C), 177.6 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+Na)^+$ $C_{14}H_{18}N_2O_7S_2Na$: 413.0453, found: 413.0457.

4.1.19. 1-[3′,5′-Bis-*S*-(methoxycarbonyl)methyl-2′-deoxy-3′,5′-dithio-β-D-*threo*-pentofuranosyl]thymine [T-3′(up),5′-bisSCH_2COOMe] 24

Compound **23**[49] (1.1 g, 2 mmol) was converted to compound **24** (0.64 g, 76%) following the method described for compound **10**. [Eluent: 40–60% of EtOAc in pet ether]. Transparent gum; ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.86 (s, 3H), 2.03–2.09 (m, 1H), 2.77–2.84 (m, 1H), 2.94–3.04 (m, 2H), 3.21–3.35 (m, 4H), 3.64–3.67 (s, 7H), 4.30–4.35 (m, 1H), 6.02–6.05 (m, 1H), 7.51 (s, 1H), 9.92 ppm (s, 1H); ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 12.4, 33.6 (CH_2), 33.7(CH_2), 33.8 (CH_2), 39.3 (CH_2), 46.2, 52.4, 52.6, 81.3, 84.1, 110.8 (C), 135.6, 150.6 (C), 164.1 (C), 170.2 (C), 170.6 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+Na)^+$ $C_{16}H_{22}N_2O_7S_2Na$: 441.0766, found: 444.0757.

4.1.20. 1-[3′,5′-Bis-*S*-(methoxycarbonyl)methyl-2′-deoxy-3′,5′-disulfonyl-β-D-*threo*-pentofuranosyl]thymine [T-3′(up),5′-bisSO_2CH_2COOMe] 25

Compound **24** (0.6 g, 1.24 mmol) was converted to compound **25** (0.48 g, 70%) following the method described for compound **14** [Eluent: 6% of MeOH in dichloromethane].White solid; mp 97–100 °C. ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.76 (s, 3H), 2.41–2.46 (m, 1H), 2.77–2.85 (m, 1H), 3.66 (s, 3H), 3.72 (s, 3H), 3.86–3.89 (m, 1H), 4.12–4.19 (m, 1H), 4.37–4.51 (m, 3H), 4.55 (d, *J* = 14.8 Hz, 1H), 4.72 (d, *J* = 15.2 Hz, 1H), 4.76–4.80 (m, 1H), 6.15–6.18 (m, 1H), 7.54 (s, 1H), 11.42 ppm (s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.8, 31.9 (CH_2), 53.1, 53.4, 54.3 (CH_2), 57.8 (CH_2), 58.6 (CH_2), 62.2, 72.1, 83.2, 110.3 (C), 136.1, 150.7 (C), 163.5 (C), 164.0 ppm (2 C); HRMS (ESI$^-$), *m*/*z* calcd for $(M-H)^-$ $C_{16}H_{21}N_2O_{11}S_2$: 481.0587, found: 481.0564.

4.1.21. 1-[3′,5′-Bis-*S*-(carboxymethyl)-2′-deoxy-3′,5′-disulfonyl-β-D-*threo*-pentofuranosyl]thymine [T-3′(up),5′-bisSO_2CH_2COOH] 26

Compound **25** (0.05 g, 0.11 mmol) was converted to compound **26** (0.035 g, 75%) following the method described for compound **21** [Eluent: 30–80% of MeOH in chloroform]. Yellow hygroscopic solid; ^{1}H NMR (D_2O, 25 °C, water suppressed): δ = 1.92 (s, 3H), 2.54–2.60 (m, 1H), 3.03–3.11 (m, 1H), 4.05–4.25 (m, 5H), 4.41–4.47 (m, 1H), 4.58–4.60 (m, 1H), 4.93–4.98 (m, 1H), 6.25–6.28 (m, 1H), 7.70 ppm (s, 1H); ^{13}C NMR (DMSO-d_6): δ 12.7, 32.0 (CH_2), 54.5 (CH_2), 57.9 (CH_2), 58.7 (CH_2), 62.3, 72.1, 83.2, 110.3 (C), 136.0, 150.7 (C), 162.9 (C), 163.4 (C), 164.0 ppm (C); HRMS (ESI$^-$), *m*/*z* calcd for $(M-H)^-$ $C_{14}H_{17}N_2O_{11}S_2$: 453.0274, found: 453.0296.

4.1.22. 1-[3′,5′-Bis-*S*-(carboxymethyl)-2′-deoxy-3′,5′-dithio-β-D-*threo*-pentofuranosyl]thymine [T-3′(up),5′-bisSCH_2COOH] 27

Compound **24** (0.1 g, 0.24 mmol) was converted to compound **27** (0.056 g, 60%) following the method described for compound **22** [Eluent: 5–80% of MeOH in chloroform]. Yellow solid; mp 115–120 °C; ^{1}H NMR (D_2O, 25 °C): δ = 2.20 (s, 3H), 2.36–2.44 (m, 1H), 2.99–3.06 (m, 1H), 3.15–3.17 (m, 2H), 3.38–3.44 (m, 4H), 3.83–3.87 (m, 1H), 4.60–4.65 (m, 1H), 6.24–6.27 (m, 1H), 7.96 ppm (s, 1H); ^{13}C NMR (D_2O, 25 °C): δ = 11.8, 33.4 (CH_2), 37.1 (CH_2), 37.4 (CH_2), 38.1 (CH_2), 45.7, 81.2, 84.8, 111.2 (C), 137.9, 151.9 (C), 166.8 (C), 177.6 (C), 177.9 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+Na)^+$ $C_{14}H_{18}N_2O_7S_2Na$: 413.0453, found: 413.0462.

4.1.23. 5′-Deoxy-5′-*S*-[(methoxycarbonyl)methylthio]thymidine 29

Compound **28**[33] (1.5 g, 3.8 mmol) was converted to compound **29**[53] (0.95 g, 76%) following the method described for compound **10**. [Eluent: 0–3% of MeOH in $CHCl_3$]. White solid; mp 117–120 °C; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.78 (s, 3H), 2.01–2.06 (m, 1H), 2.16–2.21 (m, 1H), 2.80–2.91 (m, 2H), 3.41 (q, *J* = 20.0 Hz, 2H), 3.61 (s, 3H), 3.81–3.85 (m, 1H), 4.13–4.16 (m, 1H), 5.35 (d, *J* = 4.4 Hz, 1H), 6.12–6.16 (m, 1H), 7.45 (s, 1H), 11.29 ppm (br s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.6, 33.7 (CH_2), 34.7 (CH_2), 38.5 (CH_2), 52.5, 72.9, 84.2, 85.7, 110.2 (C), 136.5, 150.9 (C), 164.1 (C), 170.9 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+Na)^+$ $C_{13}H_{18}N_2O_6SNa$: 353.0783, found: 353.0784.

4.1.24. 5′-Deoxy-5′-*S*-[(methoxycarbonyl)methylsulfonyl]thymidine (T-5′SO_2CH_2COOMe) 30

Compound **29** (0.9 g, 2.75 mmol) was converted to compound **30** (0.77 g, 70%) following the method described for compound **14**. [Eluent: 2–8% of MeOH in $CHCl_3$]. White solid; mp 195–200 °C; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.78 (s, 3H), 2.04–2.09 (m, 1H), 2.22–2.29 (m, 1H), 3.60–3.85 (m, 4H), 3.77–3.85 (m, 1H), 4.09–4.11 (m, 1H), 4.24–4.27 (m, 1H), 4.31–4.39 (m, 2H), 5.52–5.53 (m, 1H), 6.15–6.19 (m, 1H), 7.50 (s, 1H), 11.32 ppm (s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 12.4, 37.7 (CH_2), 53.1, 56.6 (CH_2), 58.4 (CH_2), 73.2, 80.3, 85.1, 110.2 (C), 136.9, 150.8 (C), 163.9 (C), 164.1 ppm (C); HRMS (ESI$^+$), *m*/*z* calcd for $(M+H)^+$ $C_{13}H_{19}N_2O_8S$: 363.0862, found: 363.0882.

4.1.25. 5′-Deoxy-5′-*S*-[(carboxymethyl)sulfonyl]thymidine (T-5′SO_2CH_2COOH) 31

Compound **30** (0.032 g, 0.09 mmol) was converted to compound **31** (0.025 g, 81%) following the method described for com-

pound **21**. [Eluent: 20–80% of MeOH in $CHCl_3$].White hygroscopic solid; ^{1}H NMR (DMSO-d_6, 25 °C): δ = 1.21 (s, 3H), 2.02–2.15 (m, 2H), 3.54–3.57 (m, 2H), 3.71–3.75 (m, 1H), 4.02–4.08 (m, 1H), 4.13–4.16 (m, 1H), 4.23–4.24 (m, 1H), 5.66 (br s, 1H), 6.13–6.17 (m, 1H), 7.65 (s, 1H), 11.30 ppm (br s, 1H); ^{13}C NMR (DMSO-d_6, 25 °C): δ = 11.9, 37.8 (CH_2), 54.5 (CH_2), 61.8 (CH_2), 72.9, 80.7, 84.5, 109.9 (C), 136.3, 150.5 (C), 163.8 (C), 164.0 ppm (C);. HRMS (ESI^+), *m*/*z* calcd for $(M+H)^+$ $C_{12}H_{17}N_2O_8S$: 349.0706, found: 349.0699.

4.2. Biophysical studies

4.2.1. Agarose gel electrophoresis assay

(i) Inhibition of RNase A was assayed qualitatively by the degradation of tRNA in an agarose gel. In this method, 20 μL of RNase A (1.51 μM) was mixed with 20 (0.07 mM), 40 (0.14 mM) and 60 μL (0.20 mM) of compounds **3**, **7**, **8**, **12**, **13**, **15**, **19**, **20**, **24**, **25** and **30** to a final volume of 100 μL and the resulting solutions incubated for 3 h. 20 μL aliquots of the incubated mixtures were then mixed with 20 μL of tRNA solution (5.0 mg/mL tRNA, freshly dissolved in RNase free water) and incubated for another 30 min. Then 10 μL of sample buffer (containing 10% glycerol and 0.025% bromophenol blue) was added to this mixture and 15 μL from each solution was taken and loaded into a 1.1% agarose gel. The gel was run using 0.04 M Tris–Acetic acid–EDTA (TAE) buffer (pH 8.0). The residual tRNA was visualized by ethidium bromide staining under UV light.

(ii) Inhibition of RNase A by compounds **16**, **21**, **22**, **26**, **27** and **31** were assayed qualitatively by the degradation of tRNA individually. In this method, 20 μL of RNase A (1.26 μM) was mixed with 20 (0.06 mM), 40 (0.12 mM), 60 μL (0.18 mM) and 80 μL (0.24 mM) of compounds **16**, **22**, **26** and **31** separately to a final volume of 100 μL and the resulting solutions were incubated for 3 h. Again, 20 μL of RNase A (2.00 μM) was mixed with 20 (0.05 mM), 40 (0.10 mM), 60 (0.15 mM) and 80 (0.20 mM) μL of compounds **21** and **26** separately to a final volume of 100 μL. Solutions were incubated for 3 h. After running the gel, residual tRNA was visualized after ethidium bromide staining under UV light.

4.3.1. Precipitation assay

Inhibition of the ribonucleolytic activity of RNase A was quantified by the precipitation assay as described by Bond[58] In this method 10 μL of RNase A (1.31 μM) was mixed with 40 μL of compounds (0.35 mM) individually to a final volume of 100 μL and incubated for 2 h at 37 °C. 20 μL of the resulting solutions from the incubated mixtures were then mixed with 40 μL of tRNA (5 mg/mL tRNA freshly dissolved in RNase A free water), 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 M 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. 50 μL of the supernatant was taken and diluted to 1 mL. The decrease in absorbance at 260 nm was measured and compared to a control set.

4.3.2. Inhibition kinetics

The inhibition of RNase A by compounds **3**, **7**, **8**, **12**, **13**, **15**, **16**, **20**–**22**, **25**–**27**, **30** and **31** were assessed individually by a spectrophotometric method as described by Anderson et al.[57] The assay was performed in 0.1 M Mes–NaOH buffer, pH 6.0 containing 0.1 M NaCl using 2′,3′-cCMP as the substrate. The inhibition constants were calculated from initial velocity data using Lineweaver–Burk plot. For the Lineweaver–Burk plot the reciprocal of initial velocity was plotted against the reciprocal of substrate concentration at a constant inhibitor concentration according to the following equation:

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

Here v is the initial velocity, [S] the substrate concentration, [I] the inhibitor concentration, K_m the Michaelis constant, K_i the inhibition constant, and V_{max} the maximum velocity. The kinetics experiments were performed with two fixed inhibitor concentrations and another in absence of inhibitor with varying substrate (2′,3′-cCMP) concentrations. The slopes from the double reciprocal plot were again plotted against the corresponding inhibitor concentrations to get inhibition constants (K_i).

5. Docking studies

The crystal structures of the protein 1FS3 (PDB entry for RNase A) was downloaded from the Protein Data Bank.[59] Water molecules and other ions present in the crystal structures were subtracted to prepare the protein PDB file for docking. The 3D structures of compounds **3**, **7**, **8**, **12**, **13**, **15**, **16**, **20**–**22**, **25**–**27**, **30** and **31** were generated in Sybyl6.92 (Tripos Inc., St. Louis, USA) and their energy-minimized conformations were obtained with the help of the MMFF94 force field using MMFF94 charges with a gradient of 0.005 kcal/mol by 1000 iterations with all other default parameters. The *FlexX* software as part of the Sybyl suite was used for docking of the ligands to the protein. The ranking of the generated solutions was performed using a scoring function that estimates the free binding energy (ΔG) of the protein–ligand complex considering various types of molecular interactions as described in Rarey et al.[60] Each docked conformation is looked upon as a 'suggestion' of how the ligand may bind with the protein. *PyMol*[61] was used for visualization of the docked conformations.

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Supplementary data

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