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Synthesis of 2-Thioorotidine and Comparison of its Unusual Instability with its Canonical Pyrimidine Counterparts

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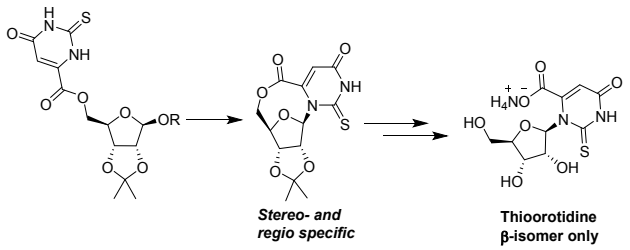
Abstract: 2-Thiopyrimidine-nucleosides exhibit properties that are interesting both from a biological/medicinal and origins of life chemistry point of view. We report here the first synthesis of the nucleoside 2-thioorotidine and our observations on its unexpected lability with respect to its *N*-glycosidic bond when compared with its corresponding canonical pyrimidine counterparts. We hypothesize that the cause of the lability of the *N*-glycosidic bond is due to the combined steric and electronic effects from the 2-thio- and the 6-carboxyl groups, a lability which could in turn be useful for further chemical transformations.



Introduction

2-Thiopyrimidines are a class of nucleosides that are naturally occurring and have been investigated primarily for their properties and use in biological and medicinal chemistry.[1] In addition, there has been a long-standing interest in the use of 2-thiopyrimidines (particularly 2-thiouridine and 2-thiocytidine) in nucleic acid systems based on the fact that they are components of transfer RNA.[2-4] Subsequently, it has been shown that inclusion of 2-thiouridine increases the stability of nucleic acid duplexes[2], which has led to investigations of the role of 2-

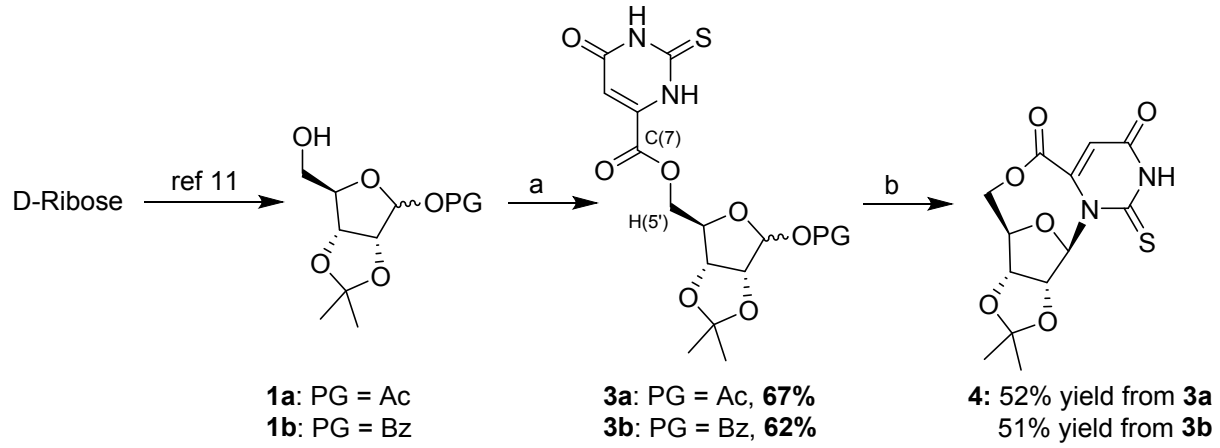
thiopyrimidines in the context of origins of life. For example, work from the Szostak lab has shown the beneficial effects of 2-thiouridine with respect to increased fidelity and rates in non-enzymatic replication of RNA.[5-7] The Sutherland[6,8] and Powner[9] labs have shown that thio derivatives of pyrimidines and purines could have played a role in producing RNA nucleosides or its precursors under plausibly prebiotic conditions. Thus, there is an increasing awareness and interest in the chemical transformation potential of the 2-thio-derivatives of the canonical nucleobases and nucleosides.[10]

In this context, we became interested in the 2-thio-derivatives of orotic acid and orotidine based on our recent work with orotic acid and orotidine containing RNA.[11-13] While 2-thioorotic acid is well precedented in the literature[14,15], 2-thioorotidine is only known as the 2-thioorotidine-5'-monophosphate which has been prepared enzymatically.[16,17] Here we describe our efforts in synthesizing the 2-thioorotidine, report on its chemical properties and compare its stability behavior with those of the corresponding canonical pyrimidine nucleosides. We find that the *N*-glycosidic bond of 2-thioorotidine is remarkably labile in water and cleanly regenerates ribose and the nucleobase, 2-thioorotic acid. This lability seems to be unusual when compared with its natural counterparts and seems to be a consequence of a combination of the carboxylic group at the 6-position and the sulfur at the 2-position.

Results and Discussions

Synthesis of 2-thioorotidine: We began our synthesis of 2-thioorotidine by employing the strategy and conditions developed in our lab previously for the successful synthesis of orotidine.[11] The approach was based on an intramolecular nucleosidation strategy to access 2-thioorotidine in a highly regio- and stereo-specific manner resting on three key steps: a) esterification of the C(6)-COOH moiety of 2-thioorotic acid with 1-*O*-acyl-2,3-*O*-isopropylidine-D-ribofuranoside (at the 5-*O*-position), b) intramolecular nucleosidation to facilitate the correct regio- and stereo-chemistry of the nucleoside and c) hydrolytic ring opening followed by acetal deprotection to furnish the 2-thioorotidine. It is important to note that in order for the intramolecular nucleosidation to work (as noted in our earlier work)[11], a cyclic protecting group at the 2',3'-*O*-positions rigidifying the furanose sugar frame work was necessary. The intramolecular nucleosidation was unsuccessful when using the acetate or benzoyl protecting groups at the 2'- and 3'-positions,.[11]

We therefore prepared two derivatives, 1-*O*-acetyl/benzoyl-2,3-*O*-isopropylidine-D-ribofuranoside **1a** and **1b** according to the reported procedure[11] in 4 steps starting from D-ribose. Initially we tested the esterification of this protected sugars (**1a**/**1b**) with 2-thioorotic acid (**2**) under the previously developed condition using carbonyldiimidazole (CDI), but failed to obtain the desired esterified products. We then turned to the standard *N,N'*-dicyclohexyl carbodiimide (DCC) with catalytic DMAP conditions to carry out the acid-alcohol coupling, which furnished the desired esterified products **3a** and **3b** in 67 and 62% yields respectively (Scheme 1). The formation of the thioorotate ester bond at the desired 5'-*O*-position of the sugar was further confirmed by HMBC-NMR spectroscopy by correlating the C(7) carbonyl carbon of 2-thioorotate with C-H(5') proton of ribofuranose sugar (Supporting Figures S3 and S7).



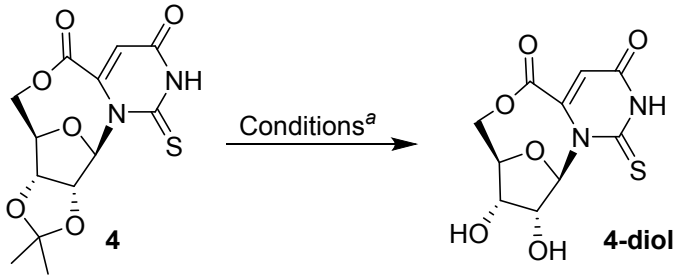
Scheme 1: Synthesis of 2-thioorotidine lactone: (a) thioorotic acid (**2**, 1.1 eq,.), DCC, DMAP, DMF, 0 °C to rt, 2 h, (b) BSA (2.2 eq.), MeCN (0.010 M), rt 1 h followed by $SnCl_4$ (0.5 eq.), 60 °C, overnight.

Next, the ester derivatives **3a** and **3b** were subjected to Vorbrüggen-Hilbert-Johnson (VHJ) nucleosidation[18] condition to obtain 5'-*O*-lactone **4**. Again, following the previously reported condition[11] for the orotidine derivative using TMSOTf was not successful in providing the desired product for the thio-derivative. Changing the Lewis acid activator to tin(IV)chloride from TMSOTf seemed to do the trick, delivering the title product 5'-*O*-lactone (**4**) in 52% yield.

With the desired 2-thioorotidine 5'-*O*-lactone (**4**) in hand and having successfully addressed two of the three key steps we were confident about the remaining pathway, the hydrolytic opening of lactone ring followed by acetonide deprotection. We adopted one of the previous protocols developed for orotidine synthesis,[11] which involves acetonide deprotection with $In(OTf)_3$ followed by methanolysis with NaOMe/MeOH to generate the methyl ester of 2-

thioorotidine, which upon subsequent hydrolysis with aq. NaOH would provide the desired 2-thioorotidine. However, in the case of the thio-derivative **4**, the three-step protocol proved problematic as we observed only the formation of 2-thioorotic acid (**2**) after the final step (confirmed by ^{1}H-NMR and ESI-mass analysis), indicating that de-pyrimidation of 2-thioorotic acid derivative **4** had taken place (Supporting Figures S40-S41). With this unexpected result, we decided to move step-wise through the synthesis to understand the issues at hand. We first investigated the option of deprotecting the acetonide ring of compound **4** to see if we could form the corresponding **4-diol** under acidic conditions. However, we again observed only glycosidic bond cleavage or decomposition of the starting material **4** (Table 1, For ^{1}H NMR see Supporting Figure S43).

Table 1: Optimization for the deprotection of the acetonide of 2-thioorotidine 5'-lactone.

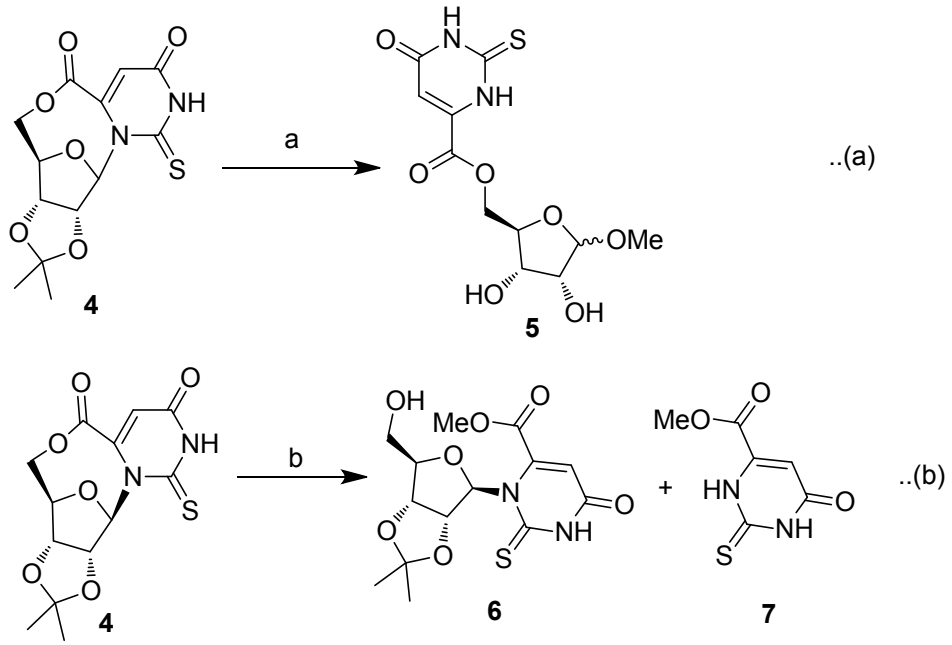


Entry	Condition	Time (h)	Temperature	Observation
1.	1 M HCl (aq.)	1	rt	N-glycosidic bond cleavage
2.	60% TFA (aq.)	1	rt	Decomposition of starting material
2.	10% TFA (aq.)	1	rt	Decomposition of starting material
4.	$In(OTf)_3$ in MeCN:H_2O	1	rt to 40 °C	Decomposition of starting material
5.	pTSA (0.5 eq.) in MeOH	24 h	50 °C	N-glycosidic bond cleavage[b]

[a]10 mg of 2-thioorotidine 5'-lactone (**4**) was dissolved in 0.3 mL of the indicated solvent in a small reaction vial. The reaction mixtures were analyzed by TLC (direct spotting) and NMR (0.2 mL reaction aliquots in 0.5 mL of D_2O). [b]The product of *N*-glycosidic bond cleavage (from entry 5) was isolated (Scheme 2a).

When we tested organic acids such as pTSA in methanolic conditions (entry 5, Table 1), the reaction (by TLC) showed complete consumption of starting material forming a new product without the usual decomposition patterns observed in the previous reactions. But, when the isolated product was analyzed by ^{1}H- and ^{13}C-NMR, it was found to be 1-methoxy-2,3-OH-5'-*O*-thioorotyl-D-ribofuranose **5** (Table 1 entry 5; Scheme 2a). This once again indicated that the *N*-

glycosidic bond in **4** was undergoing cleavage readily under the acidic conditions. For comparison, the acetonide of the 5'-*O* lactone of orotidine (the 2-oxo version of **4**) was deprotected under 60% aq. TFA to afford the corresponding 2',3'-diol in 80% yield,[11] indicating that there was something atypical with the 2-thio derivative **4** in terms of the *N*-glycosidic bond lability.

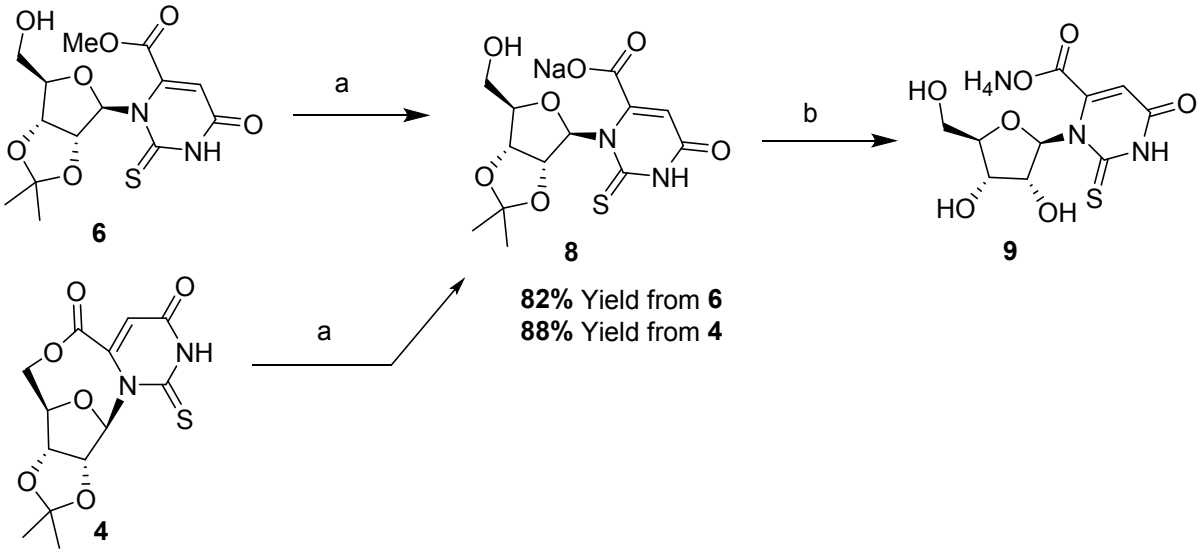


Scheme 2: Acetonide deprotection and methanolysis of 5'-*O*-lactone **4** leading to cleavage of the *N*-glycosidic bond. Conditions (a) *p*TSA (0.5 eq.), MeOH, 50 °C, 24 h. (b) 1 M NaOMe (0.2 eq.), MeOH, rt, 24 h.

Therefore, we modified the strategy to first open the lactone ring followed by acetonide deprotection to get the desired 2-thioorotidine, with the hope that relieving the strain (induced by the lactone ring) might increase the stability of the *N*-glycosidic bond. Accordingly, we attempted to generate the methyl ester of 2-thioorotidine (**6**) by treating 2-thioorotidine 5'-*O*-lactone (**4**) with 1.0 M NaOMe in methanol; However, depyrimidination to (**7**) was still the dominant pathway (based upon TLC and NMR analysis, Supporting Figure S42), The depyrimidination was further increased by silica gel chromatography (even after the silica gel was treated with 0.1% triethylamine), and we isolated the acetonide protected 2-thioorotidine methyl ester (**6**) in only 27% yield along with 41% of 2-thioorotic acid methyl ester (**7**) (Scheme 2b). Unfortunately, acetonide deprotection of the methyl ester of 2-thioorotidine (**6**) using

pTSA/methanolic condition afforded only 2-thioorotic acid methyl ester (**7**). Exploring other mineral acids such TFA, HCl, AcOH in place of pTSA again led to either decomposition of starting material **6** or depyridimination to give 2-thioorotic acid methyl ester.

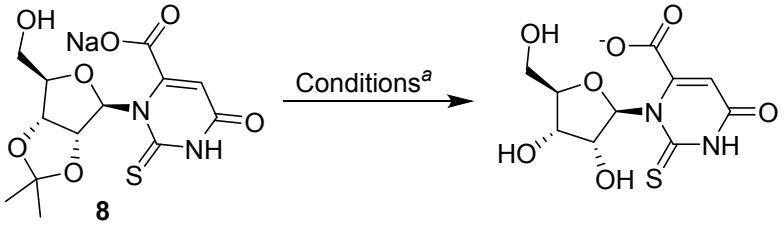
The failure to successfully deprotect the acetonide moiety of 2-thioorotidine 5'-*O*-lactone (**4**), led us to consider that increasing the electron density on the nucleobase (by forming the anionic carboxylate group by increasing the pH) may decrease the chance of depyrimidination. Also, since such processes are thought to be catalyzed by protonation of the nucleobase,[19,20] we reasoned that increasing the pH may slow down the depyrimidination process. Therefore, we proposed to make sodium salt of the acetonide protected methyl ester of 2-thioorotidine **6** (Scheme 3). Accordingly, we treated the methyl ester of 2-thioorotidine (**6**) with sodium hydroxide and were gratified to find that the reaction afforded the desired sodium salt of 2,3-*O*-isopropylidine 2-thioorotidine (**8**) in 82% yield. Compound **8** was also accessible, under the same condition, in one step from 2-thioorotidine 5'-*O*-lactone (**4**) in 88% yield. In both cases we did not observe the loss of the pyrimidine nucleobase which seemed to give credence to our reasoning above, further reinforced by the instability of the *N*-glycosidic bond in the methyl ester derivative **6** during isolation (Scheme 2).



Scheme 3: Hydrolysis of methyl ester of 2-thioorotidine. Condition (a) Crushed NaOH (2 eq.), MeCN:H_2O (1:1, v/v), rt, overnight. (b) 1M HCl in MeOH, rt, 6 h followed by 30% NH_4OH to pH ~9-10.

The sodium salt of 2-thioorotidine derivative **8** was then subjected to different acetonide deprotection conditions (Table 2, entries 1-6, For ^{1}H NMR see Supporting Figure S44) and the best condition was found to be the use of methanolic HCl (Table 2, entry 6). After complete conversion of starting material, the reaction mixture was basified with NH_4OH to produce the ammonium salt of **9**, which was stable to column chromatographic conditions to furnish the desired target molecule in 91% yield. The regio- and stereo- chemistry of the product was confirmed by HMBC-NMR spectroscopy (Supporting Figure S28).

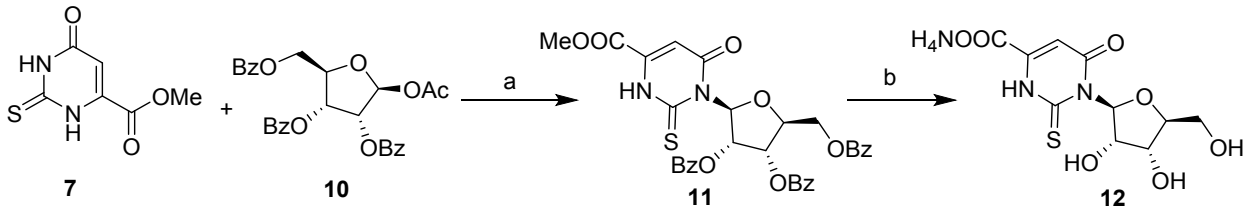
Table 2: Optimization of the deprotection of acetonide 8.



Entry	Condition	Time (h)	Temperature	Observation[b]
1.	1 M HCl (aq.)	1	rt	*N*-glycosidic bond cleavage[b]
2.	60% TFA (aq.)	1	rt	*N*-glycosidic bond cleavage[b]
2.	10% TFA (aq.)	1	rt	*N*-glycosidic bond cleavage[b]
4.	$In(OTf)_3$ in MeCN:H_2O	1	rt to 40 °C	Decomposition of starting material
5.	pTSA (0.5 eq.) in MeOH	2 h	40 °C	Decomposition of starting material
6.	1 M HCl in MeOH	6 h	rt	Trace amount of *N*-glycosidic bond cleavage observed with complete conversion of starting material[b]

[a]10 mg of 2',3'-*O*-isopropylidine--2-thioorotidine sodium salt (**8**) was dissolved in 0.3 mL of the indicated solvent in a small reaction vial. The reaction mixtures were analyzed by TLC (direct spotting) and NMR (0.2 mL reaction aliquots in 0.5 mL of D_2O). [b]Observations based upon TLC and NMR analysis.

Based on our previous work[11] we wanted to know if the direct VHJ-nucleosidation with 2-thioorotic acid would furnish the *N*-1 or the *N*-3 regioisomer of the product, since the 2-thio group may interfere as it has done with the *N*-glycosidic bond stability. It is known in case of orotidine that such direct nucleosidation approaches provide the *N*-3-regioisomer as the major product.[21-23] We had also noted in our previous work[11] that even protecting the *N*-3-position (as *N*(3)-benzoyl) of the orotic acid methyl ester still afforded the *N*-3 isomer (isoorotidine), and none of the *N*(1)-orotidine was formed by direct nucleosidation.



Scheme 4: Direct intermolecular nucleosidation of 2-thioorotidine methyl ester **7**. Conditions: (a) BSA (2.0 eq.), MeCN, 50 °C, 30 minutes followed by TMSOTf (1.0 eq.), 50 °C, 3 h. (b) Crushed NaOH (10.0 eq.), MeCN:H_2O (1:1, v/v), rt, overnight.

For the thio-derivative, we tested the same reaction for the direct nucleosidation of the methyl ester of 2-thioorotic acid (**7**) with the protected ribose sugar (1-*O*-acetyl-3,4,5-*O*-benzoyl β-D-ribofuranoside, **10**) under VHJ conditions. Under these conditions, we observed only the formation of compound **11**, which upon deprotection of benzoyl and ester groups afforded product **12** (Scheme 4). This was shown to have *N*(3)-regioselectivity as confirmed by NMR data comparison of 2-thioorotidine (**9**); iso-2-thioorotidine (**12**) showed complete dissimilarity in the chemical shift of anomeric and olefinic protons (Figure 1). Further confirmation was obtained by comparison of UV-data and HMBC-correlation studies by correlating the H-1' proton with thiocarbonyl (C-2) and carbonyl carbon (C-4) of 2-thioorotic acid (Supporting Figures S37-S39). Thus, the intramolecular nucleosidation strategy provides the desired *N*(1)-2-thioorotidine with a *β*-linkage, which may be useful in the context of our ongoing prebiotic and origins of life studies.

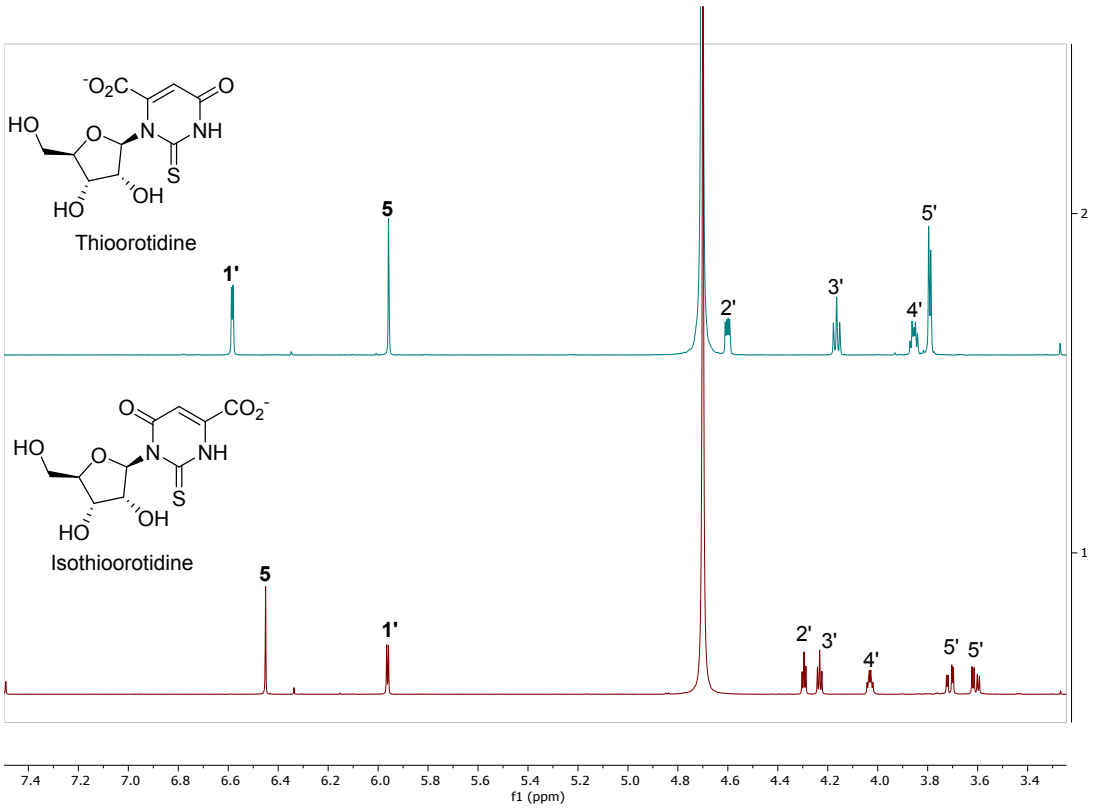


Figure 1: ^{1}H-NMR comparison of 2-thioorotidine (**9**) with iso-2-thioorotidine (**12**) in D_2O at 600 MHz

Studies on the instability of 2-thioorotidine: Even following the successful protocol reported for orotidine,[11] the unexpected lability of the *N*-glycosidic bond of 2-thioorotidine was problematic. The lability of the *N*-glycosidic bond is unusual when compared to not only orotidine but also to other thionucleosides. For example, 2-thiouridine is a stable compound, and has been studied extensively in RNA oligonucleotides.[2-7] It is also known that 2'-deoxy-2'-mercaptouridine is fairly stable in neutral or slightly acidic aqueous solution at room temperature.[24] Therefore, the relative instability of the 2-thioorotidine (**9**), which has the combination of the 6-carboxylic acid (of orotidine), 2-thio and the 2'-hydroxyl groups (of thiouridine) was surprising. Therefore, we undertook an investigation to learn how unstable the 2-thioorotidine derivative is compared to its pyrimidine counterparts, orotidine, 2-thiouridine and uridine. We subjected each of the nucleosides to a variety of aqueous pH conditions and followed their stability on a reverse phase HPLC using UV-detection. Accordingly, we prepared the aqueous solution of 2-thioorotidine **9** in water (unbuffered, pH~6.2) and in 10 mM sodium phosphate buffer at pH 3.0, 6.0, 7.0, 8.4 and 12.5. In order to compare the unusual behavior of 2-

thioorotidine, we also prepared the same aqueous solutions of orotidine, 2-thiouridine, uridine and iso-2-thioorotidine (**12**) and analyzed the samples at different intervals of time via reverse phase HPLC. As evident from the HPLC traces (Figure 2), the *N*-glycosidic bond in 2-thioorotidine is labile in acidic or neutral solution forming 2-thioorotic acid, but relatively stable in highly alkaline media (Figure 2b). The *N*-glycosidic bond is cleaved under acidic conditions (pH 3) with about 30% of 2-thioorotidine **9** remaining, while at an alkaline pH (12.5) 80% of 2-thioorotidine is still present after 2 weeks. (Figure 3a) The other three nucleosides, orotidine, thiouridine and uridine were found to be stable at all pH ranges with no discernible decomposition under similar conditions, as evidenced by the HPLC traces (Supporting Figures S51-S68). From the HPLC chromatograms, it is clear that the stability of 2-thioorotidine increases with increasing pH suggesting that the depyrimidination is probably taking place via a protonation on the nucleobase (perhaps at *N*-1).

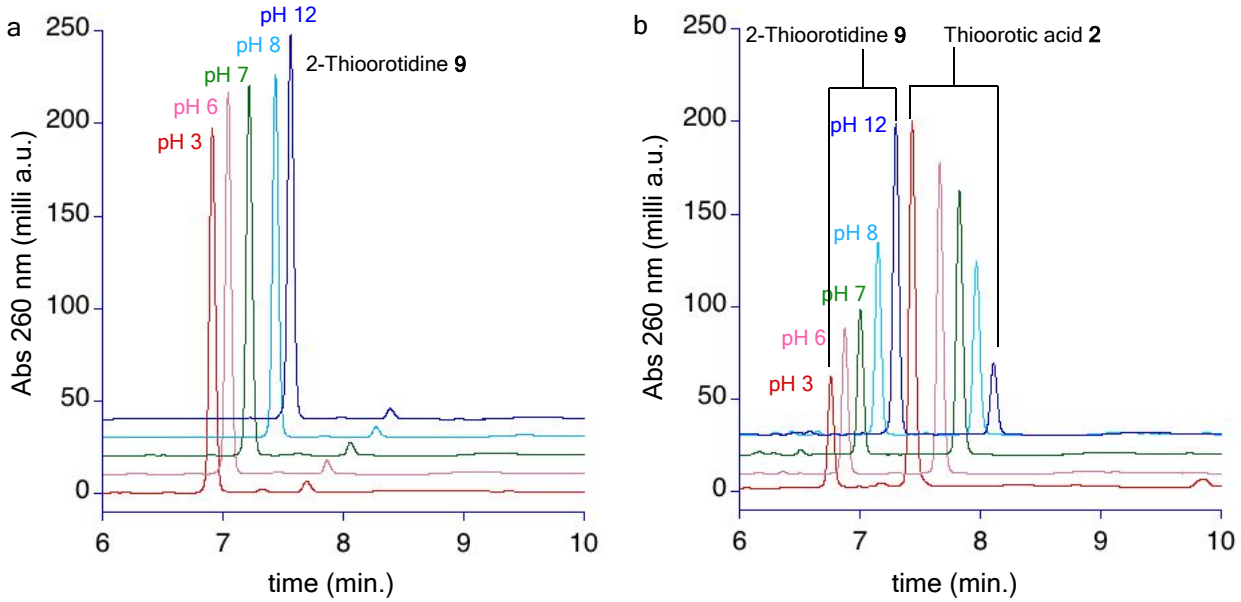


Figure 2: HPLC traces of 2-thioorotidine (~ 30 μM) at different pH values in 10 mM sodium phosphate. (a) HPLC traces of 2-thioorotidine at t = 0 h at unbuffered (pH ~ 6.2), pH 3.0, 6.0, 7.0, 8.4 and 12.5 in sodium phosphate buffer. (b) HPLC traces of 2-thioorotidine after 14 days (336 h) at unbuffered (pH ~ 6.2), pH 3.0, 6.0, 7.0, 8.4 and 12.5 in sodium phosphate buffer. pH adjustment was carried out by addition of 0.1 N HCl or 0.1 N NaOH.

The fact that under even basic conditions, 2-thioorotidine is still decomposing is perhaps suggestive of the extent to which even minimal proton concentration is enough for catalyzing this reaction. For example, when compound **4** was treated with sodium methoxide in methanol,

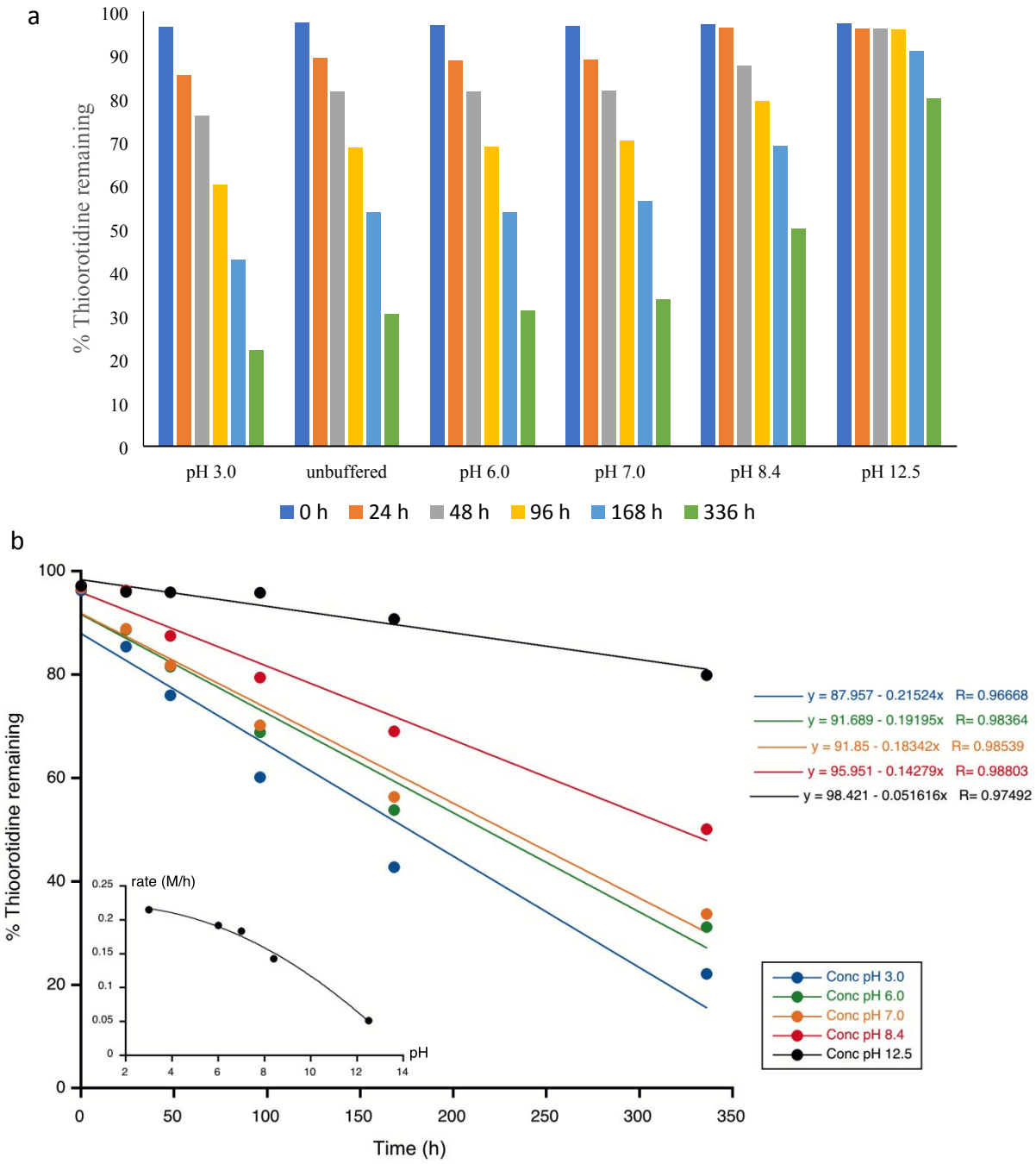


Figure 3: (a) Percentage of 2-thioorotidine remaining over different intervals of time (0 to 336 h) at different pH values (3.0 - 12.5) in sodium phosphate buffer (For HPLC analysis conditions see supporting information). Unbuffered pH was ~ 6.2. (b) The rate of change in 2-thioorotidine concentration over time (pseudo-zeroth order) and the pH-rate constant correlation (insert in 3b) obtained from HPLC data in figure 3a, showing the decrease in the deglycosylation rate of 2-thioorotidine with increasing in pH with a dramatic drop of the rate at pH 12.5.

N-glycosidic bond cleavage was minimized compared to acidic conditions, but still observed; it appears that a proton transfer that results in the transient protonated species of 2-thioorotidine

would cause this deglycosylation. Moreover, when the pH is controlled at 12.5, well above the pKa of N3(H) of 2-thioorotdine, there is a prominent stabilization when compared to acidic pH; but there is still a slow(er) and perceptible cleavage of the glycosidic bond (figure 3a and 3b insert). The linear correspondence in the change in 2-thioorotidine concentration with time over the range of pH values studied is indicative of a pseudo zeroth order rate (Figure 3b). This suggests that the proton accelerated decomposition is magnifying other internal structural factors that are causing the inherent instability of this molecule.

As to why the *N*-glycosidic bond of 2-thioorotidine (versus the other nucleosides) is highly labile, we hypothesize that it must be a combination of effects of steric and electronics from both the C(6)-CO_2H and the C(2)=S group (Figure 4). With regards to the electronic effects, the replacement of the C(2)=O with a C(2)=S is known to change the preferred protonation sites with a strong preference for C(2)=S site when compared to uridine (where there is equal preference for 2,4H tautomer and C(4)=O protonation), and that a protonation at the C=S site leads to a strong preference for the *anti*-conformation around the nucleosidic bond.[25,26] This phenomenon alone cannot be the cause of instability, since 2-thiouridine is found to be quite stable under comparable conditions. Therefore, the C(6)-COOH group must also play a role (keeping in mind orotidine is quite stable under these conditions as well). Thus, it is reasonable to conclude that it is not one or the other, but a combination of the two (the C=S and the COOH group) that is responsible for the bond instability: while the C=S increases the *anti*-conformation,[2,27] it has to contend with the steric hindrance caused by the C(6)-COOH group.[12] This combination of the increased size of sulfur atom (versus the oxygen atom) plus the steric clash induced by the COOH group, weakens the *N*-glycosidic bond (Figure 4). Thus, the protonation (of the C=S bond) at lower pH values enhances the lability of the glycosidic bond in 2-thioorotidine (Figure 4). This behavior is similar to the instability experienced by many of the pyrimidine nucleosides that are attached at *N*(3)-position of the pyrimidine nucleobases, where there is steric hindrance from the carbonyl groups at the C-2 and C-4 positions. Support for such an assertion comes from the observation that iso-2-thioorotidine (**12**) was significantly more labile than 2-thioorotidine (**9**) under mildly acidic conditions (pH 6.0) and glycosidic bond cleavage proceeded relatively rapidly (1.6x, after 24 h) (Supporting Figures S47 versus S71). Further work on iso-2-thioorotidine was limited as it begins to decompose immediately once purified and isolated.

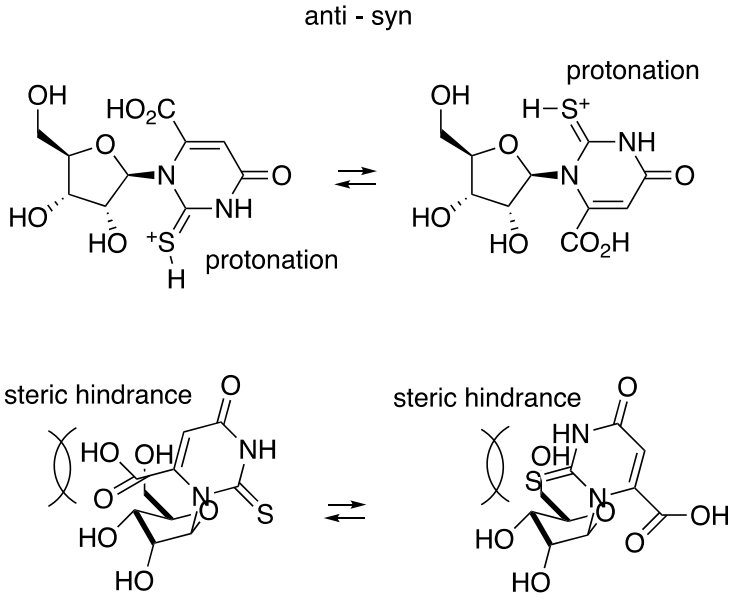


Figure 4: Postulated steric and electronic factors that may be contributing to the weakening of the *N*-glycosidic bond in 2-thiouridine. Protonation of the C=S bond typically favors the anti-conformation to relieve steric interactions between the large sulfur atom and the 5' OH.[25,26] However in 2-thioorotidine, this anti conformation is also strained by a steric clash between the C(6)carboxylate and the 5' hydroxyl.[12]

In conclusion, we have developed a successful intramolecular nucleosidation strategy and a synthetic protocol that affords us regioselectively the *N*(1)-2-thioorotidine in good yield. However, this thionucleoside was found to have unusual glycosidic bond lability, which we attribute to the combined effect of the C(6)-COOH group and the C(2)=S moiety. Further investigations (theoretical as well as experimental) are ongoing to understand the exact nature of the contributions leading to this unusual instability. We propose that such lability could be of use in a prebiotic context as recently demonstrated by the work of Sutherland and co-workers[8] in a trans-nucleosidation approach affording access to other nucleosides, an endeavor that is ongoing in our laboratory.

Experimental Section

Thioorotic acid was purchased from *Sigma Aldrich (CAS # 6953-78-2)*. 4-Dimethylamino pyridine (DMAP), *N,N'*-Dicyclohexylcarbodiimide (DCC) and Bis(trimethylsilyl)acetamide (BSA) were purchased from *Acros Organics*. Tin(IV)Chloride was purchased from *Sigma Aldrich.* Anhydrous solvents were purchased from *EMD Chemicals*. Unless otherwise noted, all experiments were performed under a nitrogen atmosphere. Thin layer chromatography (TLC) was performed on silica gel 60 Å F254 from *Angela Technologies*, and it was visualized by UV

lamp and/or a stain solution of phosphomolybdic acid (PMA) in ethanol. Flash column chromatography was performed on silica gel 60 Å with particle size 35-70 μm purchased from *Acros Organics*. NMR spectra were recorded on Bruker DRX-600 or AV-600 (600 MHz for ^{1}H and 150 MHz for ^{13}C). NMR Chemical shifts δ are given in ppm relative to the residual signals of tetramethylsilane in $CDCl_3$ or deuterated solvent DMSO-*d*^{6} for ^{1}H and ^{13}C NMR. Multiplicities are reported as follows: singlet (s), doublet (d), doublet of doublets (dd), doublet of triplets (dt), triplet (t), quartet (q), multiplet (m), broad singlet (bs). Mass spectra were measured with an Agilent ESI-TOF or ThermoElectron Finnigan LTQ ion trap mass spectrometer. The purity and characterization of these compounds were further established using HRMS (ESI-TOF) mass analysis. Mass spectra of all compounds were recorded in positive ion mode on a mass spectrometer in the Scripps Mass Spectrometry Center. HPLC was performed by ion-pair reverse phase HPLC (Phenomenex Aqua analytical column [250 mm x 4.6 mm (i.d.), 5 μm particle size] protected by a Phenomenex Security Guard [4 mm x 3.0 mm (i.d.)] C_{18} ODS column) on a Vanquish UHPLC purification system.

1′-*O*-Acetyl-2′,3′-*O*-isopropylidene-5′-*O*-thioorotyl-D-ribofuranoside (3a): To a solution of thioorotic acid (1.22 g, 7.10 mmol) in dry DMF (6.5 mL) was added 1-*O*-acetyl-2.3-*O*-isopropylidine-D-ribofuranoside (1.5 g, 6.45 mmol) and DMAP (79 mg, 0.64 mmol) at room temperature. The reaction was then cooled to 0 ^{o}C and a solution of DCC (1.46 g, 7.10 mmol) in 6.5 mL of dry DMF was added dropwise. After complete addition of DCC, the reaction was warmed to room temperature and stirred for additional 2 h. After complete consumption of starting material (ribofuranose sugar) as indicated by TLC, the reaction mixture was passed through a short bed of celite (to remove dicyclohexyl urea) and washed with ethyl acetate (2 x 20 mL). Filtrate was then collected and concentrated in a rotary evaporator to produce a residue, which was purified by silica gel column chromatography (40% ethyl acetate:hexane to 60% ethyl acetate:hexane) to afford **3a** as pale yellow solid. R_f = 0.42 (hexane:ethyl acetate, 1:1), Yield = 1.64 g (67%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.67 (s, 1H, H-5), 6.29 (s, 1H, H-1'), 4.79-4.77 (m, 2H, H-2', H-3'), 4.61-4.56 (m, 2H, H-4', H-5'), 4.39 (m, 1H, *J* = 10.6 Hz, 4.8 Hz, H-5'), 2.11 (s, 3H, Ac), 1.54 (s, 3H, Me), 1.38 (s, 3H, Me) ppm. $^{13}C\{H\}$ NMR (151 MHz, $CDCl_3$) δ 175.1 (C=S, thioorot.), 169.2 (Ac; C=O), 160.3 (thioorot.), 159.3 (thioorot.), 139.6 (thioorot.), 113.8 (quat. C), 108.4 (C-5), 102.0 (C-1'), 85.0 (C-2'), 84.9 (C-4'), 81.0 (C-3'), 67.2 (C-5'), 26.5 (C-

Me), 25.1 (C-Me), 21.2 (C-Ac) ppm, HRMS (ESI-TOF) calcd for $C_{15}H_{18}N_2O_8SNa$ $[M+Na]^+$ 409.0682 found 409.0692.

1′-*O*-Benzoyl-2′,3′-*O*-isopropylidene-5′-*O*-thioorotyl-D-ribofuranoside (3b): To a solution of thioorotic acid (965 mg, 5.61 mmol) in dry DMF (5.0 mL) was added 1-*O*-benzoyl-2.3-*O*-isopropylidine-D-ribofuranoside (1.5 g, 5.09 mmol) and DMAP (62 mg, 0.51 mmol) at room temperature. The reaction was then cooled to 0 °C and a solution of DCC (1.15 g, 5.61 mmol) in 5.0 mL of dry DMF was added dropwise. After complete addition of DCC, the reaction was warmed to room temperature and stirred for additional 2 h. After complete consumption of starting material (ribofuranose sugar) as indicated by TLC, the mixture was then passed through a short bed of celite (to remove dicyclohexyl urea) and washed with ethyl acetate. The filtrate was then collected and concentrated in a rotary evaporator to produce a residue, which was purified by silica gel column chromatography (eluted with 40% ethyl acetate:hexane to 60% ethyl acetate:hexane) to afford **3b** as pale yellow solid. R_f = 0.30 (hexane:ethyl acetate, 1:1), Yield = 1.41 g (62%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.71 (bs, 1H, NH), 12.31 (bs, 1H, NH), 7.92 (dd, J = 8.4 Hz, 1.4 Hz, 2H, Bz), 7.68 (tt, J = 7.3 Hz, 1.3 Hz, 1H, Bz), 7.52 (dd, J = 8.3 Hz, 7.4 Hz, 2H, Bz), 6.31 (s, 1H, H-5), 6.27 (s, 1H, H-1'), 5.08 (dd, J = 5.9 Hz, 1.1 Hz, 1H, H-2'), 5.01 (d, J = 5.9, 1H, H-3'), 4.68 (td, J = 6.0 Hz, 1.1 Hz, 1H, H-4'), 4.47 (dd, J = 11.6, 5.9 Hz, 1H, H-5'), 4.35 (dd, J = 11.6 Hz, 6.3 Hz, 1H, H-5'), 1.47 (s, 3H, Me), 1.34 (s, 3H, Me) ppm. ^{13}C{H} NMR (151 MHz, DMSO-d_6) δ 176.6 (thioorot. C=S), 164.8 (Bz, C=O), 160.9 (thioorot.), 159.7 (thioorot.), 141.7 (thioorot.), 134.3 (Bz), 129.7 (Bz), 129.4 (Bz), 129.2 (Bz), 112.6 (quat. C), 108.7 (thioorot. C-5), 103.4 (C-1'), 85.2 (C-4'), 84.9 (C-2'), 81.2 (C-3'), 66.6 (C-5'), 26.7 (C-Me), 25.2 (C-Me) ppm, HRMS (ESI-TOF) calcd for $C_{20}H_{21}N_2O_8S$ $[M+H]^+$ 449.1019 found 449.1012.

2′,3′-*O*-Isopropylidene-2-thioorotidine 5′-lactone (4): To a solution of 1′-*O*-acetyl-2′,3′-*O*-isopropylidene-5′-*O*-thioorotyl-*D*-ribofuranoside **3a** (1.28 g, 3.33 mmol) in anhydrous MeCN (333 mL, 0.01M) under a nitrogen environment, was added BSA (1.8 mL, 7.33 mmol) at room temperature and the mixture was stirred for an additional 1 h. Then, $SnCl_4$ (195 μL, 1.66 mmol) was added at room temperature and the reaction mixture was transferred to preheated oil bath at 60 °C. The reaction was then stirred overnight until completion of the reaction as indicated by TLC. Solid $NaHCO_3$ was added to the reaction mixture and stirred for 30 minutes at room temperature. Reaction was then filtered through sintered funnel and solvent was concentrated in

rotary evaporator to produce a residue, which was further purified by silica gel column chromatography using CH_2Cl_2:acetone as eluent (95:5 to 90:10) to afford **4** as white solid. R_f = 0.28 (Hexane:Ethyl Accetate, 1:2), Yield = 564 mg (52%). ^{1}H NMR (600 MHz, DMSO-d^6) δ 6.21 (s, 1H, H-5), 6.17 (s, 1H, H-1'), 5.28 (d, J = 6.0 Hz, 1H, H-2'), 4.74 (dd, J = 10.7 Hz, 5.3 Hz, 1H, H-4'), 4.62-4.58 (m, 2H, H-3', H-5'), 4.20 (t, J = 12.0 Hz, 1H, H-5'), 1.45 (s, 3H, Me), 1.29 (s, 3H, Me) ppm. ^{13}C NMR (151 MHz, DMSO-d^6) δ 174.9 (thioorot. C=S), 164.7 (thioorot. C-7), 159.3 (thioorot.), 144.0 (thioorot.), 111.6 (quat. C), 107.2 (C-5), 97.8 (C-1'), 87.2 (C-2'), 85.9 (C-4'), 79.2 (C-3'), 65.6 (C-5'), 26.1 (C-Me), 24.6 (C-Me) ppm, HRMS (ESI-TOF) calcd for $C_{13}H_{15}N_2O_6S$ $[M+H]^+$ 327.0651 found 327.0653.

1′-*O*-methyl-5′-*O*-thioorotyl-D-ribofuranoside (5): 2-Thioorotidine 5'-lactone **4** (250 mg, 0.77 mmol was dissolved in anhydrous MeOH (10 mL) under nitrogen environment and was added *para*-toluene sulfonic acid (66 mg, 0.38 mmol) at room temperature and reaction mixture was then heated at 50 ^{o}C and stirred for overnight. Next day, after completion of the reaction as indicated by TLC, solvent was removed in rotary evaporator and the residue thus obtained was purified by silica gel column chromatography using CH_2Cl_2:acetone (95:5 to 80:20) and afforded **5** as white solid. R_f = 0.28 (CH_2Cl_2:acetone, 8:2), Yield = 78 mg (32%). ^{1}H NMR (600 MHz, DMSO-d^6) δ 12.76 (bs, 1H, NH), 12.26 (bs, 1H, NH), 6.36 (s, 1H, H-5), 5.19 (d, J = 6.0 Hz, 1H, OH, D_2O exchangeable), 5.07 (d, J = 6.0 Hz, 1H, OH, D_2O exchangeable), (4.66 (s, 1H, H-1'), 4.48 (dd, J = 11.7 Hz, 3.0 Hz, 1H, H-5'), 4.25 (dd, J = 11.7 Hz, 4.9 Hz, 1H, H-5'), 4.10-4.03 (m, 2H, H-4', H-3'), 3.78-3.77 (m, 1H, H-2'), 3.20 (s, 3H, OMe) ppm. ^{13}C{H} NMR (151 MHz, DMSO-d^6) δ 176.7 (thiooro. C=S), 161.2 (thioorot.), 159.9 (thioorot.), 142.0 (thioorot.), 108.8 (C-5), 108.4 (C-1'), 79.8 (C-2'), 74.5 (C-4'), 71.2 (C-3'), 67.0 (C-5'), 54.9 (OMe) ppm, HRMS (ESI-TOF) calcd for $C_{11}H_{15}N_2O_7S$ $[M+H]^+$ 319.0600 found 319.0604.

2′,3′-*O*-isopropylidene-2-thioorotidine methylester (6): To a solution of 2-thioorotidine 5'-lactone **3** (462 mg, 1.42 mmol) in anhydrous MeOH (50 mL) was added 1.0 M NaOMe solution (280 µL, 0.28 mmol) prepared by dissolving 1.17 g of sodium metal in 50.8 mL of dry MeOH dropwise at room temperature and the reaction mixture was warmed to 30 ^{o}C and stirred for overnight, after which complete consumption of starting material was indicated by TLC. By evaporating the solvent in rotary evaporator over silica gel, the residue was adsorbed on silica gel and purified by column chromatography using CH_2Cl_2:acetone as eluent (95:5 to:20) to afford **6** as white foam. The product was further washed with diethyl ether to remove other impurities

present. R_f = 0.24 (CH_2Cl_2:acetone, 8:2), Yield = 137 mg (27%), ^{1}H NMR (600 MHz, DMSO-d^6) δ 6.77 (d, J = 3.1 Hz, 1H, H-1'), 6.33 (s, 1H, H-5), 5.12 (dd, J = 7.1 Hz, 3.0 Hz, 1H, OH, D_2O exchange-able), 5.01 (t, J = 5.5 Hz, H-2') 4.49 (t, J = 6.7 Hz, 1H, H-3'), 3.85 (s, 3H, OMe), 3.81 (td, J = 6.3 Hz, 4.6 Hz, 1H, H-4'), 3.55 (ddd, J = 11.7 Hz, 5.5 Hz, 4.6 Hz, 1H, H-5'), 3.47 (ddd, J = 11.8 Hz, 6.4 Hz, 5.4 Hz, 1H, H-5') ppm. ^{13}C{H} NMR (151 MHz, DMSO-d^6) δ 176.9 (thiooro. C=S), 163.3 (thioorot.), 158.6 (thioorot.), 144.0 (thioorot.), 114.5 (quart. C), 109.7 (C-5), 94.3 (C-1'), 85.4 (C-2'), 84.4 (C-4'), 79.5 (C-3'), 60.3 (C-5'), 53.6 (OMe), 27.1 (C-Me), 25.3 (C-Me) ppm, HRMS (ESI-TOF) calcd for $C_{14}H_{19}N_2O_7S$ $[M+H]^+$ 359.0913 found 359.0908.

Also, thioorotic acid methyl ester was isolated from the above column chromatography as pale-yellow solid. Yield 108 mg (41%). R_f = 0.60 (CH_2Cl_2:acetone, 8:2).

Methyl Thioorotic Acid (7): Thioorotic acid (2.0 g, 11.6 mmol) was suspended in 100 mL of dry MeOH at room temperature and HCl gas (5 g, produced from 5 g, NaCl and 5 mL H_2SO_4) was bubbled into the suspension. The reaction mixture was transfer to preheated oil bath at ~ 80 ^{o}C and refluxed for 24 h. The solvent was evaporated in rotary evaporator and to the residue was added saturated aq. $NaHCO_3$ and the aqueous layer was extracted with 3 x 100 mL of ethyl acetate. Combined organic layers were collected and washed with sat. NH_4Cl (aq.) and dried over Na_2SO_4 and concentrated to get **7** as pale yellow solid. Yield 788 mg (36%). R_f = 0.60 (CH_2Cl_2:acetone, 8:2). ^{1}H NMR (600 MHz, DMSO-d^6) δ 12.73 (bs, 1H, NH), 12.27 (bs, 1H, NH), 6.33 (s, 1H, H-5), 3.86 (s, 3H, OMe) ppm. ^{13}C{H} NMR (151 MHz, DMSO-d^6) δ 180.1 (thioorot. C=S), 166.3 (thioorot.), 163.9 (thioorot.), 153.2 (thioorot.), 104.6 (C-5), 52.0 (C-OMe) ppm. HRMS (ESI-TOF) calcd for $C_6H_7N_2O_3S$ $[M+H]^+$ 187.0177 found 187.0176.

2′,3′-*O*-isopropylidene-2-thioorotidine sodium salt (8)

Method A: To a solution of acetonide protected 2-thioorotidine methyl ester, **6** (50 mg, 0.14 mmol) in 3 mL of ACN:H_2O (1:1) mixture, was added crushed NaOH (11 mg, 0.28 mmol) at room temperature, and the reaction was allowed to stir overnight. After complete consumption of starting material as indicated by TLC, the solvent was evaporated in a rotary evaporator and the residue thus obtained was purified by silica gel column chromatography using CH_2Cl_2:MeOH:NH_4OH (~30%) as eluent (95:5:0.1 to 80:20:0.2) to afford **8** as white solid, (R_f = 0.28 in 85:15:0.1 CH_2Cl_2:MeOH:NH_4OH (~30%)) Yield = 42 mg (82%).

Method B: To a solution of 2-thioorotidine lactone, **4** (500 mg, 0.153 mmol) in 20 mL of ACN:H_2O (1:1) mixture, was added crushed NaOH (122 mg, 3.06 mmol) at room temperature,

and the reaction was allowed to stir for overnight. After complete consumption of starting material as indicated by TLC, the solvent was evaporated in a rotary evaporator and the residue thus obtained was purified by silica gel column chromatography using CH_2Cl_2:MeOH:NH_4OH (~30%) as eluent (95:5:0.1 to 80:20:0.2) to afford **8** as white solid, (R_f = 0.28 in 85:15:0.1 CH_2Cl_2:MeOH:NH_4OH (~30%)) Yield = 492 mg (88%). ^{1}H NMR (600 MHz, D_2O) δ 6.78 (s, 1H, H-1'), 6.01 (s, 1H, H-5), 5.23 (dd, *J* = 7.6 Hz, 2.5 Hz, 1H, H-2'), 4.68 (m, 1H, H-3'), 4.00 (q, *J* = 6.4 Hz, 1H, H-4'), 3.83 (dd, *J* = 12.0 Hz, 7.2 Hz, 1H, H-5'), 3.75 (dd, *J* = 12.2 Hz, 4.6 Hz, 1H, H-5'), 1.54 (s, 3H), 1.33 (s, 3H) ppm. ^{13}C{H} NMR (151 MHz, D_2O) δ 177.1 (thioorot. C=S), 163.3 (thioorot.), 160.0 (thioorot.), 156.8 (thioorot.), 113.5 (quat. C), 102.3 (C-5), 94.4 (C-1'), 86.0 (C-2'), 84.4 (C-4'), 79.0 (C-3'), 60.4 (C-5'), 27.3 (C-Me), 25.4 (C-Me) ppm, HRMS (ESI-TOF) calcd for $C_{13}H_{16}N_2O_7SNa$ $[M+Na]^+$ 367.0576 found 367.0571.

2-Thioorotidine ammonium salt (9): To a small round bottom flask containing 200 mg of 2,3-*O*-acetonide protected 2-thioorotidine sodium salt (**8**) was added 20 mL of 1 M HCl in MeOH at room temperature and the reaction mixture was stirred for six hours. After complete consumption of starting material as indicated by TLC, the reaction mixture was basified with the addition of ammonium hydroxide solution to pH ~9-10. The solvent was then co-evaporated with ethanol (2 x 10 mL) and the residue thus obtained was dissolved in minimum amount of methanol and absorbed onto silica gel, which was then purified by column chromatography using dichloromethane: methanol:NH_4OH (~30%) (2:1:0.1 to 1:1:0.1) as eluent to afford **9** as white solid. R_f = 0.32 (^{i}PrOH:H_2O:NH_4OH (~30%), 17.5:2:0.5). Yield 157 mg (91%). ^{1}H NMR (600 MHz, D_2O) δ 6.58 (d, *J* = 3.6 Hz, 1H, H-1'), 5.96 (s, 1H, H-5), 4.68-4.52 (m, 1H, H-2'), 4.16 (t, *J* = 7.6 Hz, 1H, H-3'), 3.86 (dt, *J* = 8.4 Hz, 5.0 Hz, 1H, H-4'), 3.79 (d, *J* = 5.0 Hz, 2H, H-5'), ppm. ^{13}C{H} NMR (151 MHz, DMSO-d^6) δ 177.1 (thioort. C=S), 167.3 (thioort. 7C=O), 162.4 (thioort. 4C=O), 153.8 (thioorot.), 104.4 (C-5), 96.7 (C-1'), 83.9 (C-4'), 73.1 (C-3'), 68.2 (C-2'), 61.1 (C-5') ppm, HRMS (ESI-TOF) calcd for $C_{10}H_{11}N_2O_7SNa_2$ $[M+2Na]^+$ 349.0082 found 349.0068.

***N*(3)-(2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl)-methyl thioorotate (11)**: To a suspension of methyl thioorotate (**7**) (250 mg, 1.34 mmol) in dry acetonitrile (30 mL) was added BSA (*N,O*-Bistrimethyl silylacetamide, 0.65 mL, 2.68 mmol) at room temperature. The reaction mixture was then heated at 50 °C for 30 minutes and became homogeneous. After 30 minutes the reaction mixture was cooled to room temperature. 1-*O*-Ac-2,3,5-*O*-Bz-β-D-ribofuranose (**10**) (677 mg,

1.34 mmol) and TMSOTf (0.24 mL, 1.34 mmol) were added and the reaction was stirred for an additional 3 h at 50 °C. Progress of the reaction was monitored by TLC and after 3 h complete consumption of sugar was observed, and the reaction was quenched by the addition of solid $NaHCO_3$ (100 mg). The solvent was concentrated in a rotary evaporator. The residue thus obtained was purified by silica gel column chromatography using ethyl acetate:hexane as eluent (1:5 to 1:1) to afford **11** as off-white foam. R_f = 0.28 (hexane:ethyl accetate, 1:1), Yield = 429 mg (51%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.12-8.10 (m, 2H, Bz), 8.04-8.02 (m, 2H, Bz), 7.94-7.92 (m, 2H, Bz), 7.62-7.54 (m, 3H, Bz), 7.45 (q, *J* = 8.0 Hz, 4H, Bz), 7.38-7.36 (m, 2H, Bz), 6.98 (s, 1H, H-5), 6.42 (d, *J* = 3.0 Hz, 1H, H-1'), 6.11 (dd, *J* = 5.1 Hz, 3.1 Hz, 1H, H-2'), 6.03 (dd, *J* = 6.6 Hz, 5.1 Hz, 1H, H-3'), 4.83 (dt, *J* = 6.5 Hz, 3.9 Hz, 2H, H-4'), 4.79 (dd, *J* = 12.2 Hz, 3.6 Hz, 1H, H-5'), 4.61 (dd, *J* = 12.2 Hz, 4.1 Hz, 1H, H-5'), 3.80 (s, 3H, OMe) ppm. ^{13}C{H} NMR (151 MHz, $CDCl_3$) δ 165.7, 164.8, 163.6, 163.4, 159.5, 152.2, 133.3, 133.2, 132.9, 129.5, 129.4, 129.3, 128.9. 128.3, 128.2, 128.1, 128.1, 128.0, 113.5, 85.9, 80.4, 75.2, 71.0, 62.9, 52.6 ppm, HRMS (ESI-TOF) calcd for $C_{32}H_{26}N_2O_{10}S$ $[M+Na]^+$ 653.1206 found 653.1206.

Iso-2-thioorotidine ammonium salt (12): 250 mg (0.40 mmol) of *N*(3)-(2′,3′,5′-tri-O-benzoyl-β-D-ribofuranosyl)-methyl thioorotate (**10**) was dissolved in 20 mL of 1:1 MeCN:H_2O in a small round-bottom flask at room temperature. Crushed NaOH (160 mg, 4 mmol) was added and the reaction mixture was stirred for overnight. After complete consumption of starting material as indicated by TLC, the solvent was then co-evaporated with ethanol (2 x 10 mL) and residue thus obtained was dissolved in minimum amount of methanol and absorbed onto silica gel, which was then purified by column chromatography using dichloromethane:methanol:NH_4OH (~30%) (2:1:0.1 to 1:1:0.1) as eluent to afford **12** as off-white solid. R_f = 0.28 (^{i}PrOH:H_2O:NH_4OH, 17.5:2:0.5). Yield 71 mg (59%). ^{1}H NMR (600 MHz, D_2O) δ 6.45 (s, 1H, H-5), 5.96 (d, *J* = 4.2 Hz, 1H, H-1'), 4.30 (t, *J* = 4.6 Hz, 1H, H-2'), 4.23 (t, *J* = 5.3 Hz, 1H, H-3'), 4.03 (td, J = 5.3, 3.4 Hz, 1H, H-4'), 3.71 (dd, *J* = 12.6, 3.4 Hz, 1H, H-5'), 3.61 (dd, *J* = 12.7, 5.0 Hz, 1H, H-5') ppm. ^{13}C{H} NMR (151 MHz, DMSO-d^6) δ 172.7 (thioort. C=S), 170.0 (thioort. 7C=O), 164.4 (thioort. 4C=O), 148.1 (thioorot.), 107.4 (C-5), 86.3 (C-1'), 84.2 (C-4'), 74.8 (C-3'), 70.1 (C-2'), 60.8 (C-5') ppm, HRMS (ESI-TOF) calcd for $C_{10}H_{13}N_2O_7S$ $[M+H]^+$ 305.0443 found 305.0440.

Associated Content

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Copies of ^{1}H, $^{13}C\{H\}$ NMR and HRMS spectra from pages S2-S26. Also, HPLC data from pages S26-S43.

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The manuscript was written through contributions of all authors.

Notes

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