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Lability of Glycosidic Linkages of 2'-Thio-ribonucleosides

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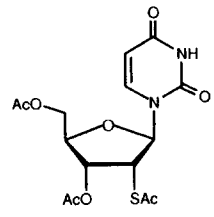
Abstract: 2'-Thiouridine **2**, 2'-thioadenosine **5** and 2'-thiocytidine **8** undergo glycosidic cleavage in aqueous solution at 25°C at and above pH 6.5. The glycosidic linkages of [1-β-D-(2-thioarabinofuranosyl)]-adenine **7** and the dimeric disulfides **12a**, **12b** and **12c**, derived from the 2'-thio-ribonucleosides **2**, **5** and **8**, respectively, are stable under the same neutral and mildly basic conditions. 2'-Thioadenosine **5** and particularly [1-β-D-(2-thioarabinofuranosyl)]-adenine **7** are more stable than 2'-deoxyadenosine to acid-catalyzed hydrolysis.

2'-Thiouridine **2**, which was the first 2'-thio-ribonucleoside described in the literature, was obtained[1] by Imazawa *et al.* in virtually quantitative yield by treating its 2'-*S*-acetyl-3',5'-di-*O*-acetyl derivative **1** with a large excess of 1.0 mol dm^{-3} potassium hydroxide in ethanol-water (1:1 v/v) at 10°C for 30 min. The latter workers also found[1] that uracil **3** was obtained as a major product when the triacetate **1** was treated "with ammonia or metallic alkali at high or moderate temperature". However these authors also reported[1] that isolated 2'-thiouridine **2** was "fairly stable in neutral or slightly acidic aqueous solution at room temperature". When the tetrabenzoyl derivative **4** of 2'-thioadenosine **5** was heated with sodium methoxide in methanol solution (see Scheme 1 and discussion below), no 2'-thioadenosine **5** was detected in the products[2] but adenine was obtained. In contrast with this result, the *arabino*-diastereoisomer **7** of 2'-thioadenosine **5** was obtained[3] in satisfactory yield when its *S*-acetyl derivative was treated with ammonia in methanol solution.



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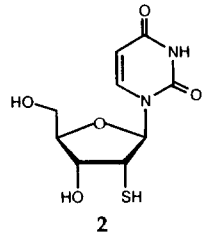

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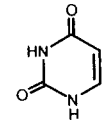

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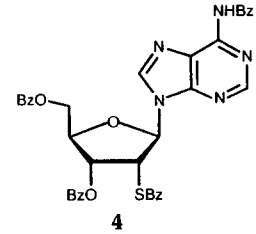

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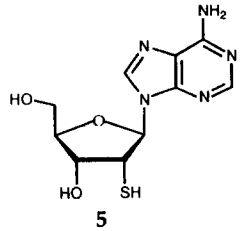

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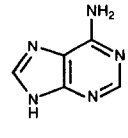

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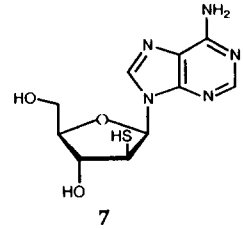

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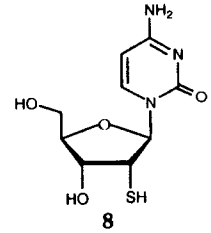

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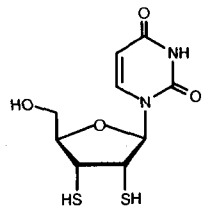
We have recently developed[4,5] what we believe to be a general synthesis of 2'-thio-ribonucleosides in which the 2'-thiol function is protected with an acid-labile protecting group that is removed in the final step. This approach has led not only to an alternative synthesis of 2'-thiouridine[4] **2** but also to the successful synthesis both of 2'-thiocytidine[4] **8** and 2'-thioadenosine[5] **5**. While previous attempts[2,6] to prepare 2'-thioadenosine **5** had all failed, a prior claim had been made for the successful synthesis[7] of 2'-thiocytidine **8**. However, the reported ^1H and ^{13}C NMR spectra[7] of the latter putative 2'-thiocytidine differed from the NMR spectroscopic data[4] that we obtained. In any case, the extreme ease with which 2'-thiocytidine **8** undergoes aerial oxidative dimerization (see below) must cast further doubt on validity of the earlier synthesis[7]. We originally adopted a strategy involving acidic unblocking conditions in order to avoid the possibility of the 2'-thio-ribonucleosides undergoing oxidative dimerization[8] to give the corresponding disulfides. However, it is clear that our approach to the synthesis of 2'-thio-ribonucleosides has the additional advantage that the possibility of base-promoted cleavage of the glycosidic linkages is also avoided.



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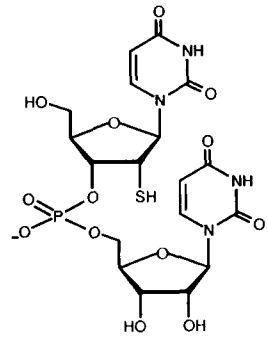

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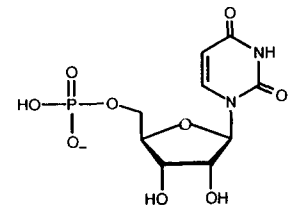

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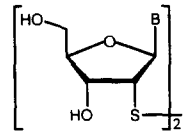
The base-lability of the glycosidic linkages of 2'-thio-ribonucleosides recently became particularly apparent to us following two independent studies. First, when 2',3'-dithiouridine[9] **9** was treated with *ca.* 2 mol. equiv. of triethylamine in methanol solution at room temperature for 1 h, uracil was obtained and isolated in 88% yield (see Experimental). The second study was perhaps of greater significance. We[10] and others[11-14] believe that the modification of the 2'-hydroxy functions of oligoribonucleotides might well prove to be a valuable tool in the elucidation of the mechanism of ribozyme action. As the replacement of a hydroxy by a thiol function is an obvious such modification, we undertook[10] the synthesis of 2'-thiouridylyl-(3'→5')-uridine **10**. We found[10] that the 2'-thiol function in the latter dinucleoside phosphate **10** was unable to participate in the cleavage of the internucleotide linkage either under acidic or basic conditions, or in the presence of ribonuclease A. However, the dinucleoside phosphate **10** did show[10] a tendency to undergo aerial oxidative dimerization (to give the corresponding symmetrical 2'→2'-disulfide) under acidic, neutral and basic conditions. Under basic conditions (e.g. at pH 9.0, room temperature) the dinucleoside phosphate **10** also underwent fragmentation to give uracil **3** and uridine 5'-phosphate **11** as identifiable products[10]. The latter compound **11** presumably resulted from the further decomposition of the apyrimidinic acid formed following the cleavage of the bond joining uracil to the anomeric centre adjacent to the 2'-thiol function. We subsequently found that when a *ca.* 0.004 mol dm^{-3} solution of 2'-thiouridine[1,4] **2** was allowed to stand at pH 9.0 in 0.1 mol dm^{-3} tris hydrochloride buffer at room temperature for 18 h, uracil **3** was obtained and was isolated from the products in 53% yield (see Experimental).



12 a; B = uracil - 1 - yl
b; B = adenin - 9 - yl
c; B = cytosin - 1 - yl

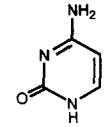


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Table 1. Reactions[a] of 2'-thio-ribonucleosides in 0.1 mol dm^{-3} tris hydrochloride buffer at 25°C

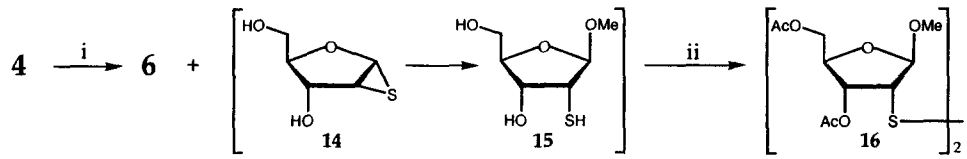
Entry No.	Substrate	pH	% Substrate[b]	% Dimer[b]	% Aglycone[b]
1	**2**	6.5	6.4 (*60.1*)	68.8 (*8.6*)	11.6 (*21.0*)
2	**2**	7.0	10.1 (*38.5*)	44.3 (*5.2*)	27.4 (*43.2*)
3	**2**	8.0	13.7 (*17.5*)	39.0 (*1.1*)	34.3 (*67.0*)
4	**2**	9.0	8.9 (*13.5*)	31.6 (*0.5*)	42.2 (*65.9*)
5	**2**	10.0	15.4 (*13.9*)	14.9 (*0.5*)	51.1 (*62.1*)
6	**8**	6.5	2.4 (*59.9*)	92.0 (*6.9*)	4.9 (*20.9*)
7	**8**	7.0	2.1 (*51.9*)	94.7 (*5.0*)	2.3 (*16.2*)
8	**8**	8.0	5.0 (*76.3*)	85.7 (*4.7*)	4.9 (*9.3*)
9	**8**	9.0	7.6 (*69.4*)	87.0 (*3.7*)	3.9 (*1.6*)
10	**8**	10.0	6.6 (*60.0*)	76.5 (*2.8*)	2.4 (*1.3*)
11	**5**	6.5	29.7 (*52.9*)	49.4 (*2.2*)	15.7 (*24.0*)
12	**5**	7.0	18.5 (*60.8*)	64.6 (*2.1*)	10.1 (*19.6*)
13	**5**	8.0	28.5 (*74.4*)	65.2 (*2.4*)	4.4 (*7.1*)
14	**5**	9.0	35.1 (*78.9*)	58.9 (*2.9*)	3.6 (*4.3*)
15	**5**	10.0	25.0 (*77.1*)	65.3 (*2.8*)	6.4 (*3.2*)

[a]Initial substrate concentrations were *ca.* 0.004 mol dm^{-3} and all reactions were allowed to proceed for 20 h. [b]Percentage estimates are based on reverse phase HPLC analyses. Experiments were carried out both in the absence and presence of 0.04 mol dm^{-3} 2-mercaptoethanol. The italicized numbers in parentheses represent the percentages obtained in the presence of 2-mercaptoethanol. In the latter experiments other unidentified products, possibly including unsymmetrical disulfides, were detected by HPLC.

These observations have prompted us to investigate the dependence of the base-promoted cleavage of the glycosidic linkages of 2'-thio-ribonucleosides on pH. Solutions (*ca.* 0.004 mol dm^{-3}) of 2'-thiouridine **2** in 0.1 mol dm^{-3} tris hydrochloride buffer (pH 6.5, 7.0, 8.0, 9.0 and 10.0) were allowed to stand at 25°C for 20 h and were then analyzed by reverse phase HPLC. It can be seen from Table 1 (entries nos. 1-5) that, in all five experiments, little substrate remained. However, while dimer[1,4] **12a** predominated at pH 6.5 - 8.0, uracil **3** was the main product at pH 9.0 and 10.0. As the experiments were carried out with only *ca.* 0.001 g (0.004

mmol) of substrate **2**, it did not prove possible to prevent the occurrence of aerial oxidative dimerization. The susceptibility of 2'-thiouridine **2** to undergo glycosidic cleavage was more clearly revealed when a *ca.* tenfold excess of 2-mercaptoethanol over substrate was added to the reaction solution to impede oxidative dimerization (Table 1, entries nos. 1-5, italicized numbers in parentheses).

In the absence of 2-mercaptoethanol, 2'-thiocytidine[4] **8** (Table 1, entries nos. 6-10) showed more tendency to undergo oxidative dimerization (see above) than 2'-thiouridine **2**, and very little cytosine **13** was obtained over the whole pH range examined. As can be seen from the experiments carried out in the presence of 2-mercaptoethanol (entries nos. 6-10, italicized numbers in parentheses), even when oxidative dimerization was largely suppressed, 2'-thiocytidine **8** showed little tendency to undergo glycosidic cleavage except perhaps at pH 6.5 and 7.0. In the absence of 2-mercaptoethanol, 2'-thioadenosine[5] **5** (entries nos. 11-15) also showed more tendency to undergo oxidative dimerization than glycosidic cleavage, and again the corresponding experiments carried out in the presence of 2-mercaptoethanol (entries nos. 11-15, italicized numbers in parentheses) confirmed that glycosidic cleavage did not occur readily, especially at pH 8.0 - 10.0. It is noteworthy that all three dimers[1,4,5] **12a-c** were stable in 0.1 mol dm^{-3} tris hydrochloride buffer at 25°C for 20 h over the whole pH range (6.5 - 10.0) examined, and in no case was the corresponding aglycone detected. Finally, while no adenine **6** was obtained when the *arabino*-compound[3,5] **7** was allowed to stand at 25°C for 20 h in 0.1 mol dm^{-3} tris hydrochloride buffer solution over the whole pH range (6.5 - 10.0), extensive oxidative dimerization was observed (see Experimental).



Scheme 1 *Reagents and conditions:* i, NaOMe, MeOH, reflux, 2 hr; ii, Ac_2O, C_6H_5N, RT

Ryan *et al.* reported[2] that when the tetrabenzoyl derivative **4** was heated, under reflux, with sodium methoxide in methanol solution (Scheme 1) and the products were acetylated after the removal of adenine **6**, the dimeric glycoside **16** was obtained in 57% isolated yield. Although they were unable firmly to establish the intermediacy of 2'-thioadenosine **5** or its 6-*N*-benzoyl derivative, the latter workers suggested[2] that adenine resulted from the internal nucleophilic attack of the released 2'-thiolate ion on the anomeric centre leading, *via* an intermediate episulfide **14** and monomeric glycoside **15**, to the isolated product **16**. In the present study, only the 2'-thio-ribonucleosides **2**, **5** and **8** and neither the dimers **12a-c** nor the *arabino*-2'-thio-nucleoside **7** decomposed to give the corresponding aglycones in the pH range 6.5 - 10.0. It is therefore reasonable to suggest that, under these conditions, the deglycosylation of the above 2'-thionucleosides **2**, **5** and **8** is accompanied by the formation of the intermediate episulfide[2] **14**. It is relevant to add that Imazawa *et al.* estimated[1] that the pK_a of the thiol function in 2'-thiouridine **2** was 7.3. What is still perhaps difficult to rationalize in connection with the data presented in Table 1 is the observed dependence of the percentage yield of aglycone on pH and on the nature of the aglycone itself.

As would be expected[15], 2'-thioadenosine **5** [$t_{1/2}$ = *ca.* 62 min in 0.01 mol dm^{-3} hydrochloric acid (pH 2.0) at 60°C] underwent acid-catalyzed cleavage of its glycosidic linkage less readily than 2'-deoxyadenosine [$t_{1/2}$ = *ca.* 22 min under the same conditions] but much more readily than adenosine. It is noteworthy that 9-[1-β-D-(2-thioarabinofuranosyl)]-adenine **7** [$t_{1/2}$ = *ca.* 42 min in 0.01 mol dm^{-3} hydrochloric acid at 100°C] was appreciably more stable to acidic hydrolysis than 2'-thioadenosine **5**. As expected, 2'-thiouridine **2** was very resistant indeed to acidic hydrolysis under the latter conditions [pH 2.0, 100°C].

EXPERIMENTAL

NMR spectra were measured at 360 MHz with a Bruker AM 360 spectrometer ; tetramethylsilane was used as an internal standard. Merck silica gel 60 F_{254} TLC plates were developed in solvent system A [chloroform-methanol (9:1 v/v)]. Liquid chromatography (HPLC) was carried out on a Jones APEX Octadecyl 5μ column which was eluted with 0.1 mol dm^{-3} triethylammonium acetate - acetonitrile mixtures. Tris hydrochloride buffer solutions were prepared by following a literature procedure[16]. The 2'-thionucleoside and disulfide substrates were all prepared by literature procedures[4,5].

*Reaction Between 2',3'-Dithiouridine **9** and Triethylamine in Methanol Solution.* - 2',3'-Dithiouridine[9] (0.10g, 0.036 mmol) was dissolved in a 0.36 mol dm^{-3} solution of triethylamine in methanol (2.0 cm^3) at room temperature. After 1 h, when TLC (system A) revealed that no starting material remained, the products were filtered and the residue was washed with ether. The dried residue (0.036 g, 88%) was identified as uracil; R_f 0.21 (system A); δ_C [$(CD_3)_2SO$] 10.3, 142.3, 151.6, 164.4.

*Decomposition of 2'-Thiouridine **2** in 0.10 mol dm^{-3} Tris Hydrochloride Buffer (pH 9.0).* - 2'-Thiouridine (0.10 g, 0.38 mmol) was dissolved in 0.10 mol dm^{-3} tris hydrochloride buffer (pH 9.0, 100 cm^{-3}) at room temperature. After 18 h, the products were concentrated under reduced pressure and the residue was chromatographed on a column of DEAE Sephadex A-25. The column was eluted with a linear gradient (from 0.01 - 0.80 mol dm^{-3} over 1.0 dm^3) of aqueous triethylammonium bicarbonate buffer (pH 7.5). The appropriate fractions were combined and concentrated under reduced pressure. Crystallization of the residue from water gave uracil (0.023 g, 53%), identical [TLC (system A), ^{13}C NMR] to authentic material.

*Behaviour of 2'-Thionucleosides **2**, **5**, **8** and **7** and Corresponding Disulfides **12a**, **12b** and **12c** in 0.1 mol dm^{-3} Tris Hydrochloride Buffer Solutions (pH 6.5 - 10.0) at 25°C.* - 0.1 mol dm^{-3} Tris hydrochloride buffer solutions were prepared. Each of the 7 substrates (**2**, **5**, **8**, **7**, **12a**, **12b** and **12c**; 0.004 mmol) was dissolved in 1.0 cm^3 of each of the 5 buffer solutions (pH 6.5, 7.0, 8.0, 9.0 and 10.0), and the solutions were placed in sealed vessels. All of the solutions were kept at 25°C for 20 h, and were then analyzed by HPLC. The HPLC column was eluted with mixtures of 0.1 mol dm^{-3} triethylammonium acetate - acetonitrile, using either isocratic or gradient elution programmes as appropriate. The results obtained with substrates **2**, **5** and **8** are given in Table 1 (entries nos. 1-5, 11-15 and 6-10, respectively). [1-β-D-(2-Thioarabinofuranosyl)]-adenine **7** was found to undergo only oxidative dimerization (52, 83, 98, 95 and 97% at pH 6.5, 7.0, 8.0, 9.0 and 10.0, respectively), and the symmetrical disulfides **12a**, **12b** and **12c** remained unchanged.

Reactions involving the three 2'-thio-ribonucleosides **2**, **5** and **8** were carried out in the same way in buffer solutions that were also 0.04 molar with respect to 2-mercaptoethanol. The results of these experiments are also given in Table 1 (entries nos. 1-5 for 2'-thiouridine **2**: entries nos 6-10 for 2'-thiocytidine **8**; entries nos. 11-15 for the 2'-thioadenosine **5**).

Acid-catalysed Hydrolysis of 2'-Deoxyadenosine, 2'-Thioadenosine **5** *and [1-β-D-(2-Thioarabinofuranosyl)]-adenine* **7**. - A solution of substrate (0.01 g) in 0.01 mol dm^{-3} hydrochloric acid (pH 2.0, 10 cm^3), contained in a sealed vessel, was heated either at 60°C (for 2'-deoxyadenosine and 2'-thioadenosine **5**) or at 100°C (for [1-β-D-(2-thioarabinofuranosyl)]-adenine **7**). Aliquots of the hydrolysate were removed after appropriate intervals of time and analyzed by HPLC. Satisfactory straight lines were obtained by plotting $\log_{10}$ (remaining substrate) against time. The half-times of hydrolysis of 2'-deoxyadenosine and 2'-thioadenosine **5** were found to be *ca.* 22 and 62 min, respectively, at 60°C; the half-time of hydrolysis of [1-β-D-(2-thioarabinofuranosyl)]-adenine **7** was found to be *ca.* 42 min at 100°C. Some concomitant oxidative dimerization both of 2'-thioadenosine **5** and [1-β-D-(2-thioarabinofuranosyl)]-adenine **7** occurred in the course of hydrolysis. The rate of hydrolysis of 2'-thiouridine **2** was too slow to measure with any degree of accuracy under the latter conditions.

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