PERKIN 1

Preparation and cleavage reactions of 3′-thiouridylyl-(3′→5′)-uridine

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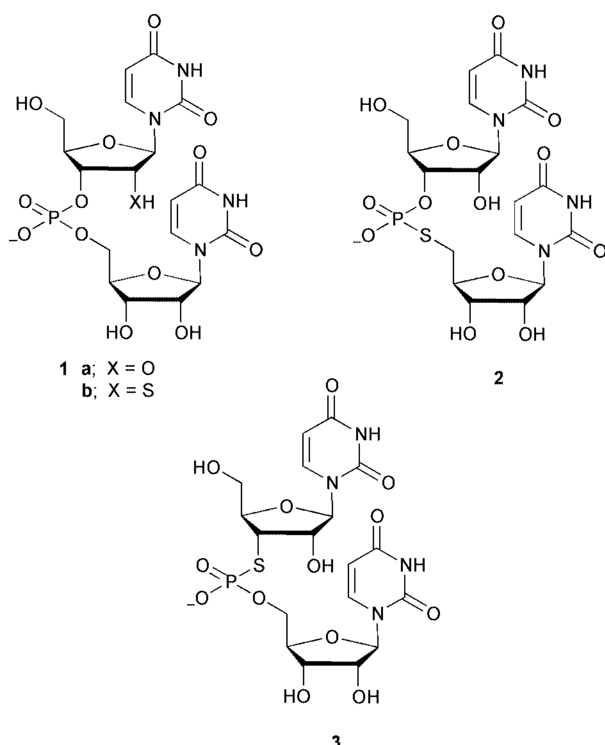
3′-Thiouridylyl-(3′→5′)-uridine [(Us)pU] **3** is prepared by coupling together the disulfide **14** and the 5′-H-phosphonate **18**, and then removing the protecting groups. (Us)pU **3** readily undergoes cleavage in 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.06) at 50 °C to give, in the first instance, uridine **4** and 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**; in glacial acetic acid at 30 °C, it rapidly undergoes cleavage in essentially the same way. The behaviour of (Us)pU **3** is compared with that of uridylyl-(3′→5′)-uridine (UpU) **1a** under the same basic and acidic reaction conditions. (Us)pU **3** and 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** are both substrates for ribonuclease A; (Us)pU **3** is a substrate for *Crotalus adamanteus* snake venom phosphodiesterase but not for calf spleen phosphodiesterase.

Introduction

The concept of antisense chemotherapy[1] has stimulated studies in the synthesis of oligodeoxyribo- and oligoribo-nucleotide analogues, and especially of analogues in which the sugar residues or internucleotide linkages are modified. Research work concerned with the elucidation of the mechanism of ribozyme action[2] has also led to the synthesis of oligonucleotide analogues. Our own studies[3] in this area have to a large extent been motivated by a long-standing interest in the fundamental chemistry of ribonucleic acids (RNAs). As part of these studies, we have examined the effect of replacing the 2′-hydroxy function of uridylyl-(3′→5′)-uridine (UpU) **1a** by a thiol function (as in **1b**). The results of this particular study proved to be relatively uninteresting in that 2′-thiouridylyl-(3′→5′)-uridine[4] **1b** does not display the equivalent of what is arguably the most characteristic property of an oligoribonucleotide in that the 2′-thiol function does not appear to interact with the internucleotide phosphodiester linkage either under basic or acidic conditions. Furthermore, 2′-thiouridylyl-(3′→5′)-uridine **1b** is not a substrate for ribonuclease A. We have also studied the effect of replacing the 5′-bridging oxygen of UpU **1a** by a sulfur atom. The properties of the resulting analogue, uridylyl-(3′→5′)-(5′-thiouridine) [Up(sU)][5] **2** are somewhat similar to those of UpU **1a** itself except that Up(sU) is very much (by a factor of ≈4 orders of magnitude) more susceptible to hydrolysis under basic conditions than is UpU. These observations have been confirmed by other workers.[6,7] In order to complete this particular group of studies, we have examined the effect of replacing the 3′-bridging oxygen of UpU **1a** by a sulfur atom to give 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] **3**. We now describe the detailed results of this last study, which has already been reported in a preliminary form.[8]



Results and discussion

3′-Thiouridylyl-(3′→5′)-uridine **3** was prepared from a nucleoside building block **14** (Scheme 1) and the triethylammonium salt of 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine 5′-H-phosphonate **18** (Scheme 2). In the preparation of compound **14**, uridine **4** was first converted (Scheme 1a) *via* its 5′-*O*-trityl-2′,3′-di-*O*-mesyl derivative **5** into the *lyxo*-epoxide[9] **6** in high overall yield. As previously reported,[9] the *lyxo*-epoxide reacted with the conjugate base of 4-methoxytoluene-α-thiol **7** to give a mixture of the isomeric sulfides **8** and **9** in the respective proportions of ≈2 : 1. The products were readily separated by chromatography on silica gel. The configuration at C-2′ of the major isomer **8** was inverted by Mitsunobu esterification[10] with 4-nitrobenzoic acid, followed by deacylation with alcoholic methylamine. When the product was heated in 80% acetic acid solution, under reflux, 3′-*S*-(4-methoxybenzyl)-3′-thiouridine **10** was obtained in ≈16% overall yield for the seven steps starting from uridine. The regio- and stereo-chemistry of compound **10** is based on ^{1}H and ^{13}C NMR spectroscopic data. It is clear from the ^{1}H COSY spectrum of compound **10** that its secondary hydroxy function (at δ 5.92) is attached to H-2′ (δ 4.01) and that the (4-methoxybenzyl)sulfanyl group is attached to H-3′ (δ 3.19). The interactions between H-6 (δ 8.02) and H-2′ and H-3′ in the NOESY spectrum of 3′-*S*-(4-methoxybenzyl)-3′-thiouridine **10**

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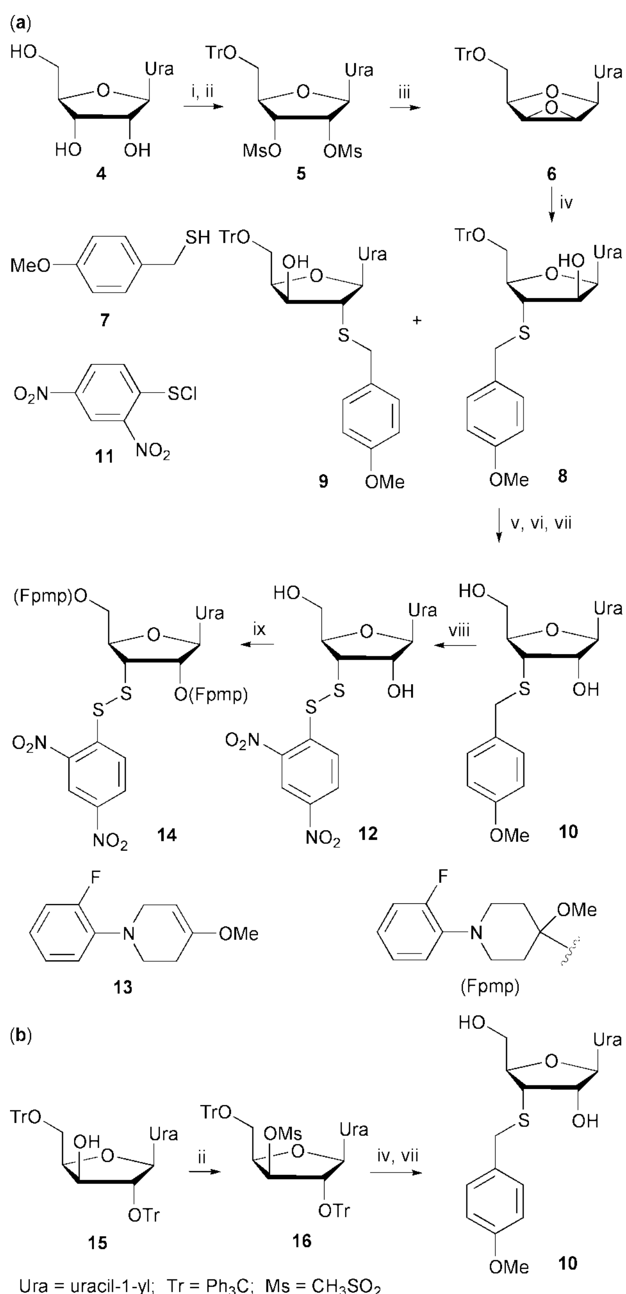
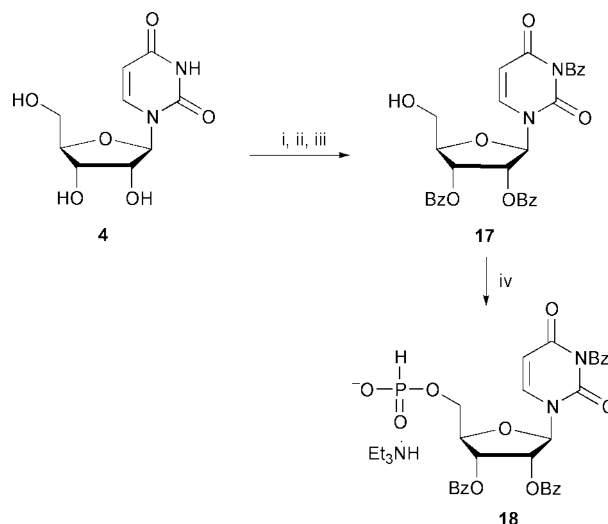


Scheme 1 *Reagents and conditions*: i, Ph_3CCl, C_5H_5N, 100 °C; ii, CH_3SO_2Cl, C_5H_5N; iii, aq. NaOH, 1,4-dioxane, 100 °C; iv, **7**, NaH, DMA, 100 °C; v, 4-nitrobenzoic acid, Ph_3P, $EtO_2CN{=}NCO_2Et$, THF, room temp.; vi, $MeNH_2$, EtOH, room temp.; vii, AcOH–H_2O (4 : 1, v/v), reflux; viii, **11**, CF_3CO_2H, CH_2Cl_2, 0 °C; ix, **13**, CF_3CO_2H, CH_2Cl_2, room temp.

provide confirmatory evidence for the configuration both at C-2′ and C-3′. Further firm evidence in support of the constitution of the latter compound **10** comes from its alternative preparation (see below) from 1-(2′,5′-di-*O*-trityl-β-D-xylofuranosyl)uracil[11] **15**. 3′-*S*-(4-Methoxybenzyl)-3′-thiouridine **10** reacted rapidly[9] with 2,4-dinitrobenzenesulfenyl chloride **11** in the presence of trifluoroacetic acid (TFA) in dichloromethane solution to give 3′-*S*-(2,4-dinitrobenzenesulfanyl)-3′-thiouridine **12**. When the latter compound **12** was treated with 1-(2-fluorophenyl)-4-methoxy-1,2,3,6-tetrahydropyridine[12,13] **13** also in the presence of TFA in dichloromethane solution, the required nucleoside building block **14** was obtained in ≈70% overall yield for the two steps starting from compound **10**. The alternative preparation (Scheme 1b) of the latter compound **10** also involved seven steps, starting from uridine. However, 1-(2′,5′-di-*O*-trityl-β-D-xylofuranosyl)uracil[11] **15**, which may be prepared from uridine in four steps, is relatively inaccessible and its conversion, *via* its 3′-*O*-mesyl derivative **16**, into 3′-*S*-(4-methoxybenzyl)-3′-thiouridine **10** proceeded in only 15% overall yield for the three steps.

The triethylammonium salt of 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine 5′-H-phosphonate **18** was prepared (Scheme 2)



Scheme 2 *Reagents and conditions*: i, Ph_3CCl, C_5H_5N, 100 °C; ii, BzCl, C_5H_5N, room temp.; iii, AcOH–water (4 : 1 v/v), reflux; iv, a, reagent prepared from PCl_3, Et_3N, 1*H*-1,2,4-triazole and THF, −35 °C; b, Et_3N, water, THF, −35 °C to room temp.

from uridine in four steps. Uridine was first converted *via* its 5′-*O*-trityl derivative into 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine[14] **17**, which was isolated in ≈48% overall yield for the three steps. Treatment of the latter compound **17** with a reagent prepared[15] from phosphorus trichloride, 1*H*-1,2,4-triazole and triethylamine in THF followed by hydrolysis with aqueous triethylamine gave the desired triethylammonium salt of the corresponding 5′-H-phosphonate **18** in ≈90% overall yield. This material, which was isolated as a colourless solid, was found by reversed-phase HPLC to be ≈99% pure.

The nucleoside building block **14** and 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine 5′-H-phosphonate **18** were coupled together by a modification of a procedure first reported by Cosstick and his co-workers[16] in the synthesis of a corresponding dinucleoside phosphorothioate in the deoxy-series. Thus the disulfide **14** and the H-phosphonate **18** were treated (Scheme 3) with chlorotrimethylsilane and triethylamine in dry dichloromethane solution at room temperature. The benzoyl protecting groups were then removed by treatment with methanolic ammonia at room temperature and the Fpmp protecting groups were also removed at room temperature by hydrolysis under mild acidic conditions.[17] The products were finally fractionated by anion-exchange chromatography to give the triethylammonium salt of 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] **3**. This material, which was isolated as a colourless solid, was characterised on the basis of ^1H and ^{31}P NMR spectroscopic data and its chemically and enzymically promoted cleavage reactions (see below); its homogeneity was established both by ^{31}P NMR spectroscopy (Fig. 1a) and reversed-phase HPLC (Fig. 1b). The chemical shift of the phosphorus resonance (δ_P [D_2O] 18.7) in the ^{31}P NMR spectrum of (Us)pU **3** (Fig. 1b) is fully consistent[5,18] with that of a phosphorothioate diester with a bridging sulfur atom.

In order to characterise the main nucleotide cleavage products (see below) of (Us)pU **3**, the preparation of 3′-thiouridine 3′-phosphorothioate **20** and 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** was undertaken (Scheme 4). 3′-*S*-(2,4-Dinitrophenylsulfanyl)-3′-thiouridine **12** (Scheme 1a) was allowed to react with tris(trimethylsilyl) phosphite.[19] Following hydrolytic work-up and fractionation of the products by anion-exchange chromatography, triethylammonium 3′-thiouridine 3′-phosphorothioate **20** was obtained and isolated as a colour-

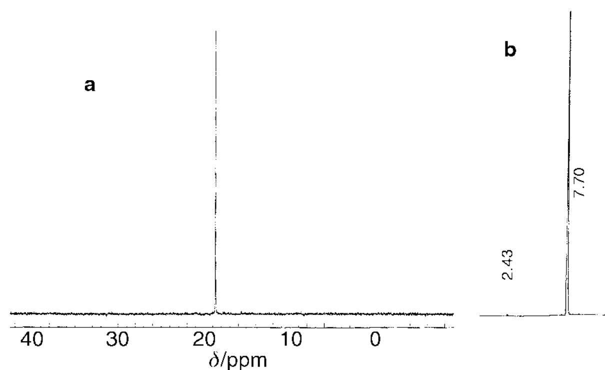
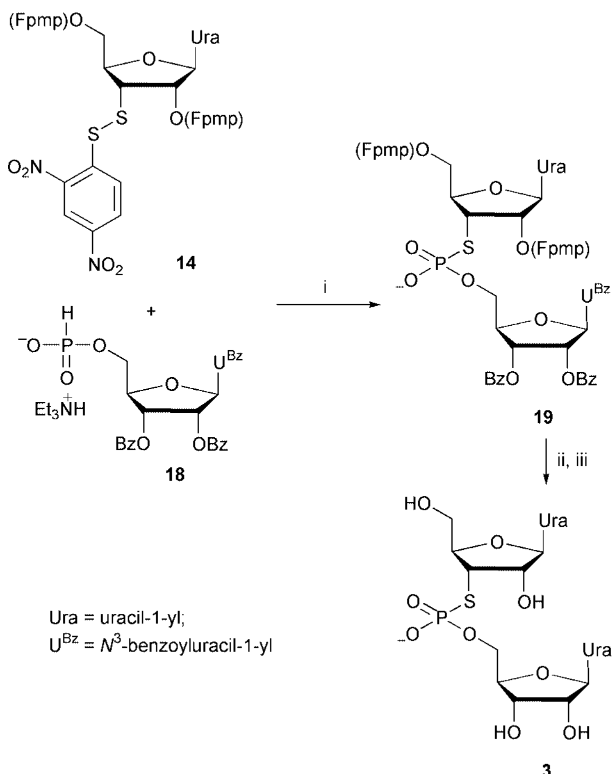


Fig. 1 (**a**) ^{31}P NMR spectrum (145.8 MHz; D_2O) of triethylammonium 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] **3**; (**b**) reversed-phase HPLC profile (programme 2) of (Us)pU **3**.



Scheme 3 *Reagents and conditions*: i, Me_3SiCl, Et_3N, CH_2Cl_2, room temp.; ii, NH_3, MeOH, room temp.; iii, AcOH–water (2:98 v/v), room temp.

less solid. This material was characterised on the basis of ^1H and ^{31}P ($\delta_P[D_2O]$ 16.4) NMR data and its homogeneity was confirmed by HPLC analysis (Fig. 2a). When an aqueous solution of 3′-thiouridine 3′-phosphorothioate **20** was treated with ethyl chloroformate and tributylamine, according to Michelson's procedure,[20] 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** was obtained. This material, which was isolated as a colourless solid following anion-exchange and silanised silica gel chromatography, was again characterised on the basis of ^1H and ^{31}P ($\delta_P[D_2O]$ 37.0) NMR data. The relative homogeneity of the cyclic phosphorothioate **21** can be seen from its reversed-phase HPLC profile (Fig. 2b).

It was important to investigate the base- and acid-catalysed hydrolysis of (Us)pU **3** and to determine whether or not it was a substrate for common phosphodiesterases. (Us)pU **3** was found to undergo hydrolysis rapidly in 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.06) at 50 °C. Pseudo-first-order kinetics were observed with a half-life ($t_{1/2}$) of *ca.* 25 min. The primary hydrolysis products (Scheme 5) were found to be uridine **4** and 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**. Under these conditions, the cyclic phosphorothioate **21** underwent further

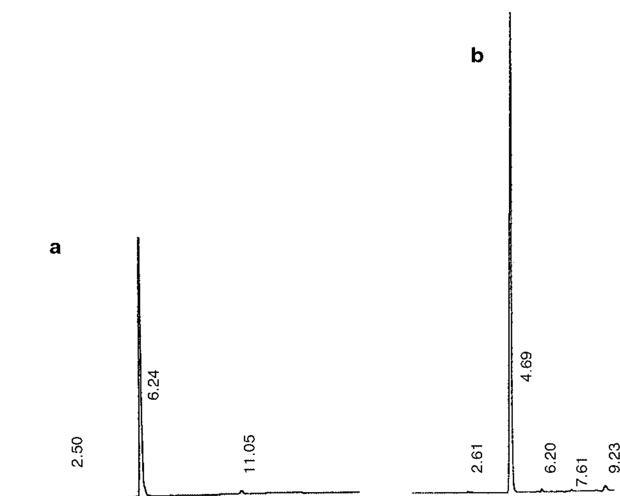
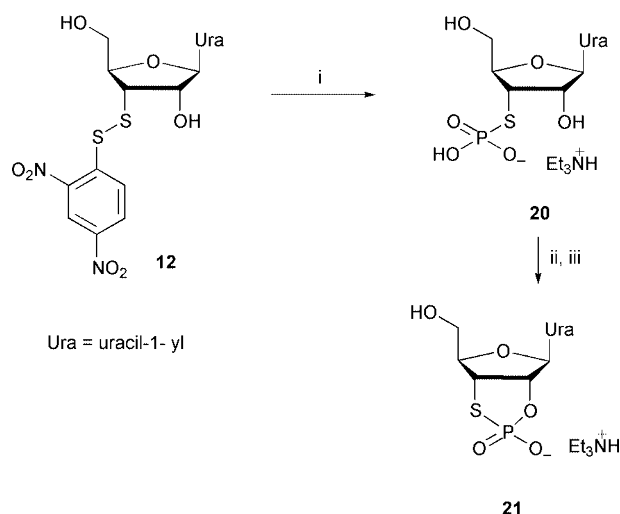
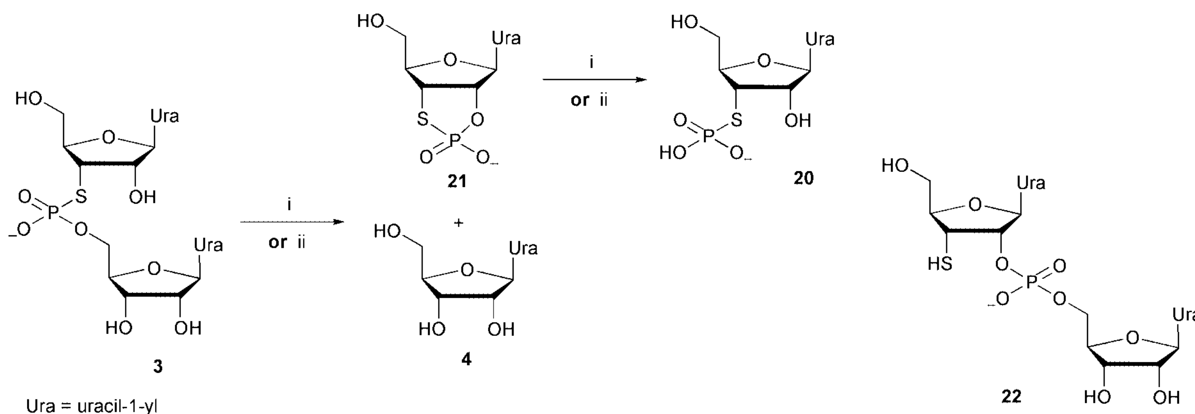


Fig. 2 Reversed-phase HPLC profiles (programme 2) of (**a**) triethylammonium 3′-thiouridine 3′-phosphorothioate **20** and (**b**) 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**.



Scheme 4 *Reagents and conditions*: i, a, Me_3SiCl, Et_3N, CH_2Cl_2, room temp.; b, $(Me_3SiO)_3P$; ii, $ClCO_2Et$, Bu_3N, water, room temp.

hydrolytic cleavage to give 3′-thiouridine 3′-phosphorothioate **20**. In a separate experiment, carried out in 0.05 mol dm^{-3} sodium glycinate buffer (pH 9.87) at 30 °C, the cyclic phosphorothioate **21** was cleanly converted into the 3′-phosphorothioate **20**. This reaction also displayed pseudo-first-order kinetics with $t_{1/2}$ *ca.* 164 min. The product composition for the hydrolysis of (Us)pU **3** after 45 min under the above reaction conditions (*i.e.* pH 10.06, 50 °C; Scheme 5, i) is illustrated in Fig. 3a. On the basis of their retention times, the four major components were identified as uridine **4** (t_R 3.37 min, 37% of total absorbance), 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** (t_R 4.83 min, 14% of total absorbance), 3′-thiouridine 3′-phosphorothioate **20** (t_R 6.27 min, 22.9% of total absorbance) and unchanged (Us)pU **3** (t_R 7.76 min, 25.3% of total absorbance). The action of 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.06) on (Us)pU **3** at 50 °C was also monitored by ^{31}P NMR spectroscopy. The reaction was carried out on a larger scale and, after 30 min, the products were neutralised and worked up. The ^{31}P NMR spectrum (D_2O) of the products is illustrated in Fig. 4a: the resonance signals at δ 37.2, 18.7 and 16.3 ppm may be assigned to 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**, (Us)pU **3** and 3′-thiouridine 3′-phosphorothioate **20**, respectively. This larger scale reaction (see Experimental section) appeared to proceed more slowly ($t_{1/2}$ > 30 min) than the reaction described above. The point of greatest interest to emerge from these results is that 3′-thiouridylyl-(3′→5′)-uridine



Scheme 5 *Reagents and conditions*: i, 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.06), 50 °C; ii, glacial acetic acid, 30 °C.

[(Us)pU] **3** undergoes base-catalysed hydrolysis at pH ≈ 10 much more rapidly than does uridylyl-(3′→5′)-uridine (UpU) **1a**. In 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.06) at 50 °C, the half-life of hydrolysis of UpU **1a** was found to be *ca.* 80–90 h. Thus, under these conditions, (Us)pU **3** undergoes base-catalysed hydrolysis at a rate approximately 200 times faster than does unmodified UpU **1a**. A similar result was subsequently reported by other workers[21,22] in connection with a study relating to 3′-thioinosylyl-(3′→5′)-uridine **27** (see below). The reason for this enhanced rate of hydrolysis is not obvious as the cleavage of (Us)pU **3** to give the cyclic phosphorothioate **21**, like the corresponding cleavage of UpU **1a**, involves a simple ester-exchange reaction without direct participation of the sulfur atom. An explanation or partial explanation for the greater lability of (Us)pU **3** is that the presence of the relatively large 3′-sulfur atom leads to a lower-energy transition state in the cyclisation reaction and to a less strained product **21**.

3′-Thiouridylyl-(3′→5′)-uridine **3** was found to decompose rapidly in glacial acetic solution at 30 °C. Precise kinetic data were not obtained but, under these conditions (Scheme 5, ii), $t_{1/2}$ for the decomposition of (Us)pU **3** was *ca.* 4 min. The product composition after 10 min is illustrated in Fig. 3b. On the basis of their retention times, the major components were identified as uridine **4** (t_R 3.30 min, 38.3% of total absorbance), 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** (t_R 4.71 min, 36.2% of total absorbance), 3′-thiouridine 3′-phosphorothioate **20** (t_R 6.19 min, 4.6% of total absorbance) and unchanged (Us)pU **3** (t_R 7.67 min, 18.6% of total absorbance). Thus the cleavage products obtained in glacial acetic acid and indeed the course of the cleavage reactions themselves (Scheme 5, ii) are qualitatively similar to those observed in pH 10.06 sodium glycinate buffer (Scheme 5, i; see also Fig. 3a). There are, however, two significant differences. First, the proportions of cyclic phosphorothioate **21** and 3′-phosphorothioate **20** obtained are different and secondly, a fifth, albeit minor product (t_R 7.51 min, 2.3% of total absorbance; Scheme 3b) is obtained in the glacial acetic acid reaction. As the latter reagent is essentially free from water, it is hardly surprising that relatively little hydrolysis of the cyclic phosphorothioate **21** occurs and therefore that the proportion of 3′-phosphorothioate **20** obtained is small. Although it has not been fully characterised, the fifth and least abundant product (Fig. 3b; t_R 7.51 min) may be 3′-thiouridylyl-(2′→5′)-uridine **22** *i.e.* the product of phosphoryl migration. There is some evidence in favour of this conclusion. First, this product appears, like the isomeric 2′-thiouridylyl-(3′→5′)-uridine[4] **1b**, to be stable under acidic conditions. Secondly, NMR spectroscopic evidence has been obtained in support of the fifth product being a phosphate rather than a phosphorothioate ester. A solution of (Us)pU **3** in glacial acetic acid was maintained at 30 °C for 25 min. The ^{31}P NMR spectrum (in D_2O) of the products is illustrated in Fig. 4b: the resonance signals at δ 37.2 (≈81%), 18.7 (≈11%) and 16.8 (≈5%) may, like the corresponding signals in Fig. 4a, be assigned to

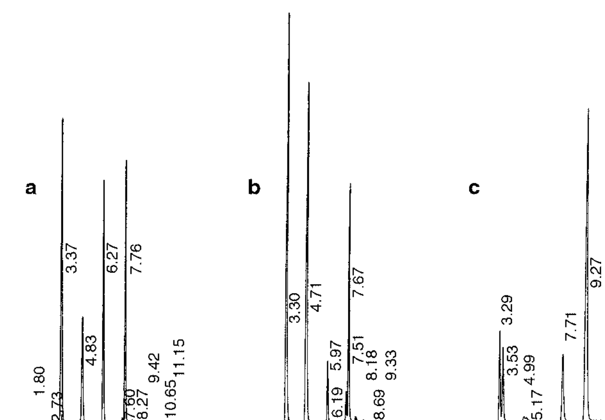
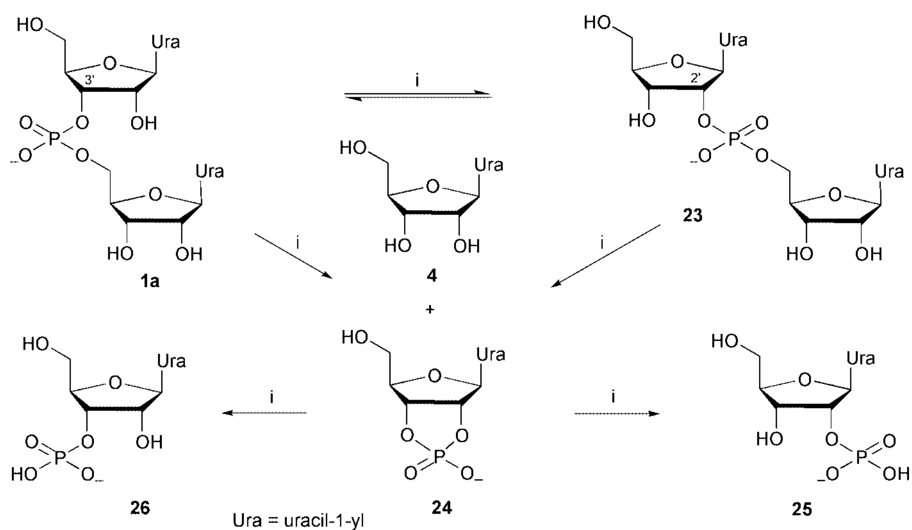


Fig. 3 (**a**) Reversed-phase HPLC profile (programme 2) of products obtained by treating 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] **3** with 0.5 mol dm^{-3} sodium glycinate buffer (pH 10.06) at 50 °C for 45 min; (**b**) reversed-phase HPLC profile (programme 2) of products obtained by treating (Us)pU **3** with glacial acetic acid at 30 °C for 10 min; (**c**) reversed-phase HPLC profile (programme 3) of products obtained by treating uridylyl-(3′→5′)-uridine [UpU] **1a** with glacial acetic acid at 30 °C for 37 min. See appropriate parts of the text for the identification of the component peaks.

3′-thiouridine 2′,3′-cyclic phosphorothioate **21**, unchanged (Us)pU **3** and 3′-thiouridine 3′-phosphorothioate **20**, respectively. The fourth resonance signal at δ −0.44 (≈3%) may possibly be assigned to 3′-thiouridylyl-(2′→5′)-uridine **22**. The phosphorus signal in the ^{31}P NMR spectrum (D_2O) of the isomeric 2′-thiouridylyl-(3′→5′)-uridine **1b** resonates[4] at δ −0.3. However, the signal at δ −0.44 (Fig. 4b) could alternatively be assigned to the phosphorus resonance of 3′-thiouridine 2′-phosphate, which would be obtained if acetic acid also promoted the hydrolytic cleavage of the P–S bond of 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**.

It has been known for many years[23] that, under acidic conditions (*e.g.* pH 1.0, 25 °C), UpU **1a** both isomerises to uridylyl-(2′→5′)-uridine **23** and undergoes hydrolytic cleavage. We now report that UpU **1a** is more stable ($t_{1/2}$ *ca.* 60 min) in glacial acetic acid solution at 30 °C (Scheme 6) than is (Us)pU **3**. The product composition after 37 min, which includes uridine **4** (t_R 3.29 min, 11.8% of total absorbance), uridine 2′,3′-cyclic phosphate **24** (t_R 3.53 min, 10.0% of total absorbance), uridine 2′(3′)-phosphates **25/26** (t_R 4.99, 5.17 min, 1.5% of total absorbance), uridylyl-(2′→5′)-uridine **23** (t_R 7.71 min, 12.8% of total absorbance) and unchanged UpU **1a** (t_R 9.27 min, 63.9% of total absorbance), is illustrated in Fig. 3c. Compared with (Us)pU **3**, UpU **1a** undergoes acetic acid-promoted cleavage to give uridine **4** and uridine 2′,3′-cyclic phosphate **24** comparatively slowly, but phosphoryl migration to give the isomeric dinucleoside phosphate **23** occurs much more readily. Indeed, it is by no means firmly established from the data described



Scheme 6 *Reagents and conditions*: i, glacial acetic acid, 30 °C.

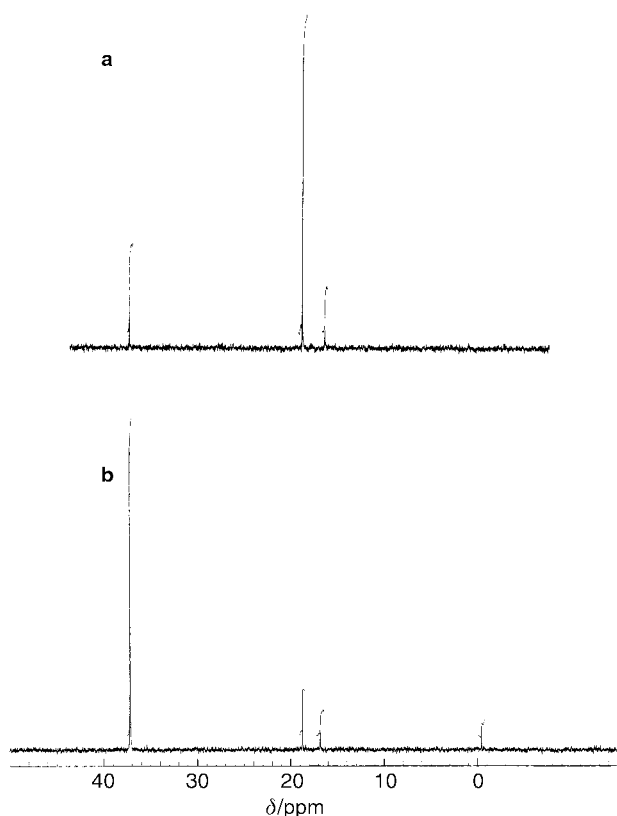
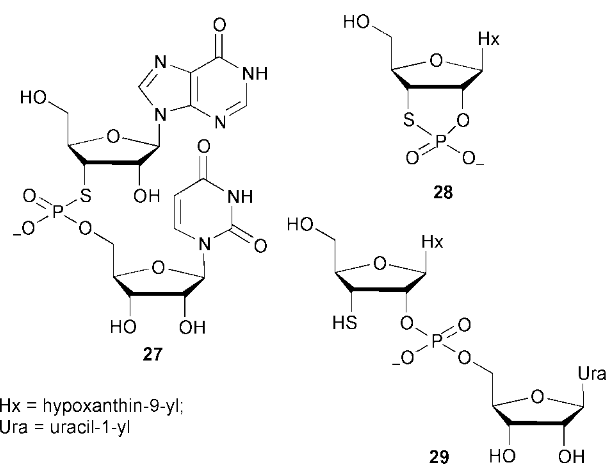


Fig. 4 ^{31}P NMR spectra (145.8 MHz; D_2O) of products obtained by treating 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] **3** (**a**) with 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.06) at 50 °C for 30 min and (**b**) with glacial acetic acid at 30 °C for 25 min.



above (Figs. 3b and 4b) that (Us)pU **3** displays any tendency whatsoever to isomerise to 3′-thiouridylyl-(2′→5′)-uridine **22** in glacial acetic acid solution. Furthermore, if isomerisation does occur (*i.e.* if the minor component with t_R = 7.51 min in Fig. 3b is 3′-thiouridylyl-(2′→5′)-uridine **22**), the isomerisation reaction is very much slower than the cleavage reaction that leads initially to 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** and uridine **4**.

In the light of the results of the above experiments relating to the action of glacial acetic acid on (Us)pU **3**, our attention was drawn to a very recent report by Elzagheid *et al.*[22] concerning the action of hydrochloric acid at 90 °C and over a range of pHs on 3′-thioinosylyl-(3′→5′)-uridine [(Is)pU] **27**. These authors reported[22] that at pH 2 and below the behaviour of (Is)pU **27** corresponds to that of UpU **1a** with regard to the comparative rates of cleavage (to give uridine and 3′-thioinosine 2′,3′-cyclic phosphorothioate **28** instead of uridine 2′,3′-cyclic phosphate **24**) and isomerisation [to give 3′-thioinosylyl-(2′→5′)-uridine **29** instead of uridylyl-(2′→5′)-uridine **23**]. Indeed these authors claimed[22], that, at pH 2, the isomerisation of (Is)pU **27** to give the (2′→5′)-dinucleoside phosphate **29** proceeded more rapidly than the cleavage reaction. It is perhaps worth noting that the reactions between 0.1 mol dm^{-3} hydrochloric acid (pH 1.0)[17,23] and UpU **1a** and glacial acetic acid and UpU are similar except that in the aqueous medium uridine 2′,3′-cyclic phosphate **24** does not accumulate but is converted into a mixture of uridine 2′- and 3′-phosphates **25** and **26**. Like Elzagheid *et al.*,[22] we shall not attempt to offer an explanation for the differences between the results obtained from the action of glacial acetic acid on (Us)pU[8] **3** and those obtained from the action of hydrochloric acid on (Is)pU **27**. However, this apparent discrepancy has encouraged us to report our own results in greater detail and to illustrate them with what we believe to be firm HPLC and ^{31}P NMR spectroscopic evidence. We also considered it important to describe in some detail the independent synthesis and characterisation of the main cleavage products (*i.e.* 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** and 3′-thiouridine 3′-phosphorothioate **20**) that were obtained both by the action of pH 10.06 sodium glycinate buffer and glacial acetic acid on (Us)pU **3**.

Finally, the action of ribonuclease A, *Crotalus adamanteus* snake venom phosphodiesterase and calf spleen phosphodiesterase on (Us)pU **3** was examined. Both (Us)pU **3** and 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** proved to be very good substrates for ribonuclease A. At 30 °C (Us)pU **3** underwent over 80% digestion in the presence of ribonuclease A in

pH 7.5 Tris hydrochloric buffer solution in 26 min to give a mixture of uridine **4**, 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** and 3′-thiouridine 3′-phosphorothioate **20**. The relative proportions of the cyclic phosphorothioate **21** and the 3′-phosphorothioate **20** were ≈1 : 2. After 146 min, the substrate had been virtually completely digested to uridine **4** and 3′-thiouridine 3′-phosphorothioate **20**. In a separate experiment, which was carried out under similar conditions, 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** underwent ≈30% ribonuclease A-promoted digestion to 3′-thiouridine 3′-phosphorothioate **20** in 5 min and complete digestion in 155 min. Perhaps unsurprisingly, (Us)pU **3** proved (see Experimental section), like UpU **1a**, to be a substrate for *Crotalus adamanteus* snake venom phosphodiesterase but, unlike UpU, not to be a substrate for calf spleen phosphodiesterase.

Experimental

^{1}H and ^{13}C NMR spectra were measured at 360.1 and 90.6 MHz, respectively, with a Bruker AM 360 spectrometer; tetramethylsilane was used as an internal standard. *J*-Values are given in Hz. ^{31}P NMR spectra were measured at 145.8 MHz with the same spectrometer; 85% orthophosphoric acid was used as an external standard. UV spectra were measured with a Perkin-Elmer model 552 spectrophotometer. Merck silica gel 60 F_{254} pre-coated plates (Art 5715 and 5642) which, unless otherwise stated, were developed in solvent system A [chloroform–methanol (9 : 1 v/v)], were used for TLC. Liquid chromatography (HPLC) was carried out on a Jones Apex Octyl 10μ column, which was eluted with 0.1 mol dm^{-3} triethylammonium acetate buffer–acetonitrile mixtures with a flow rate of 1.5 cm^3 min^{-1}: programme 1 involved a linear gradient over a period of 10 min starting with buffer–acetonitrile (70 : 30 v/v) and ending with buffer–acetonitrile (30 : 70 v/v); programme 2 involved a linear gradient over 10 min starting with buffer–acetonitrile (97 : 3 v/v) and ending with buffer–acetonitrile (80 : 20 v/v), followed by a linear gradient over 5 min ending with buffer–acetonitrile (70 : 30 v/v); programme 3 involved a linear gradient over 10 min starting with buffer–acetonitrile (97 : 3 v/v) and ending with buffer–acetonitrile (93 : 7 v/v); programme 4 involved a linear gradient over a period of 10 min starting with buffer–acetonitrile (100 : 0 v/v) and ending with buffer–acetonitrile (80 : 20 v/v). Merck Kieselgel H (Art 7729) was used for short-column chromatography; Merck silanised silica gel (Art 7719) was used for reversed-phase column chromatography. DEAE Sephadex A-25 was used for anion-exchange chromatography. Acetonitrile, tetrahydrofuran (THF) and triethylamine were dried by heating with calcium hydride, under reflux, for 3–5 h and were then distilled under atmospheric pressure; *N*,*N*-dimethylacetamide (DMA) was dried by heating over calcium hydride and was then distilled under reduced pressure; dichloromethane was dried by heating, under reflux, over phosphorus pentaoxide and was then distilled. All solvents were stored over molecular sieves (no. 4 A). Phosphorolytic enzymes were purchased from the Sigma Chemical Company.

3′-*S*-(4-Methoxybenzyl)-3′-thiouridine 10

(a) A solution of uridine **4** (14.5 g, 59.4 mmol) and chlorotriphenylmethane (18.2 g, 65.3 mmol) in dry pyridine (200 cm^3) was heated at 100 °C for 2 h. The stirred products were cooled to 0 °C (ice–water-bath) and methanesulfonyl chloride (13.8 cm^3, 0.178 mol) was added. The reactants were maintained at 4 °C for 15 h and water (20 cm^3) was then added. After 15 min, the products were evaporated under reduced pressure. The residue was dissolved in chloroform (500 cm^3) and the resulting solution was washed with saturated aq. sodium hydrogen carbonate (3 × 300 cm^3). The combined aqueous layers were back-extracted with chloroform (300 cm^3). The organic layers were combined, dried ($MgSO_4$), and evaporated under reduced pressure. The residue was dissolved in 1,4-dioxane (1400 cm^3). Aq. sodium hydroxide (2.5 mol dm^{-3}; 140 cm^3, 0.35 mol) was then added and the reaction mixture was heated under reflux. After 3 h, the products were cooled to room temperature; they were then neutralised (to pH 7) by the slow addition of glacial acetic acid and concentrated to dryness under reduced pressure. A solution of the residue in chloroform (500 cm^3) was washed first with saturated aq. sodium hydrogen carbonate (2 × 250 cm^3) and then with brine (250 cm^3). The dried ($MgSO_4$) organic layer was evaporated under reduced pressure. The residue was fractionated by short-column chromatography on silica gel: the appropriate fractions, which were eluted with chloroform–methanol (99 : 1 v/v), were evaporated under reduced pressure to give 1-(5′-*O*-trityl-2′,3′-anhydro-β-D-lyxofuranosyl)uracil[9] **6**, as a colourless foam (23.66 g); R_f 0.60 (system A); δ_H [$(CD_3)_2SO$] 3.21 (2 H, m), 4.11 (2 H, m), 4.27 (1 H, m), 5.63 (1 H, d, *J* 8.1), 6.12 (1 H, s), 7.25–7.45 (15 H, m), 7.52 (1 H, d, *J* 8.1), 11.48 (1 H, br s); δ_C [$(CD_3)_2SO$] 55.5, 55.7, 62.4, 75.9, 80.9, 86.3, 102.0, 127.2, 128.0, 128.2, 140.9, 143.4, 150.4, 163.0.

4-Methoxytoluene-α-thiol **7** (10.2 cm^3, 73.2 mmol) was added dropwise to a stirred suspension of sodium hydride (60% dispersion in mineral oil; 2.36 g, 59.0 mmol) in DMA (35 cm^3) at 0 °C (ice–water-bath). The resulting solution was stirred at room temperature for a further period of 30 min. 1-(5′-*O*-Trityl-2′,3′-anhydro-β-D-lyxofuranosyl)uracil **6** (13.84 g) was added in four portions over a period of 15 min and the stirred reactants were then heated at 100 °C. After 1 h, the products were concentrated under reduced pressure. The residue was dissolved in chloroform (500 cm^3) and the resulting solution was washed first with brine (4 × 300 cm^3) and then with water (300 cm^3). The dried ($MgSO_4$) organic layer was evaporated under reduced pressure. TLC (system A) revealed a major (R_f 0.51) and a minor (R_f 0.60) product. This mixture was fractionated by short-column chromatography on silica gel. The minor component was eluted with petroleum spirit (distillation range 40–60 °C)–ethyl acetate (4 : 1 v/v). Elution of the column with chloroform–methanol (99 : 1 v/v) and evaporation of the appropriate fractions gave 1-[3′-*S*-(4-methoxybenzyl)-3′-thio-5′-*O*-trityl-β-D-arabinofuranosyl]uracil[9] **8** as a colourless foam (9.36 g); R_f 0.51 (system A); δ_H [$(CD_3)_2SO$] 3.20 (3 H, m), 3.64 (3 H, s), 3.75 (1 H, d, *J* 13.1), 3.83 (1 H, d, *J* 13.4), 3.86 (1 H, m), 4.35 (1 H, m), 5.21 (1 H, d, *J* 8.2), 6.04 (2 H, m), 6.81 (2 H, d, *J* 8.7), 7.19 (2 H, d, *J* 8.7), 7.31 (15 H, m), 7.68 (1 H, d, *J* 8.1), 11.34 (1 H, br s); δ_C [$(CD_3)_2SO$] 33.7, 47.2, 55.0, 62.3, 76.4, 79.8, 84.0, 86.3, 100.2, 113.8, 127.2, 128.0, 128.3, 129.6, 130.0, 141.7, 143.2, 150.5, 158.2, 163.1.

The above material **8** (4.75 g), 4-nitrobenzoic acid (2.55 g, 15.3 mmol) and triphenylphosphine (4.41 g, 16.8 mmol) were dissolved in dry acetonitrile (20 cm^3) and the resulting solution was evaporated under reduced pressure. The residue was re-evaporated from its solution in dry THF (20 cm^3) and then redissolved in dry THF (30 cm^3). The stirred solution was cooled to 0 °C (ice–water-bath) and diethyl azodicarboxylate (2.64 cm^3, 16.8 mmol) was added dropwise over a period of 25 min. The stirred reactants were then allowed to warm up to room temperature. After 17 h, the products were evaporated under reduced pressure and the residue was fractionated by short-column chromatography on silica gel. The appropriate fractions, which were eluted with petroleum spirit (40–60 °C)–ethyl acetate (70 : 30 to 60 : 40 v/v), were combined, and evaporated under reduced pressure. The residue was dissolved in alcoholic methylamine (≈5 mol dm^{-3}; 10 cm^3) at room temperature. After 10 min, the products were evaporated under reduced pressure and the residue was fractionated by short-column chromatography on silica gel. The appropriate fractions, which were eluted with chloroform–methanol (99 : 1 v/v), were combined, and evaporated under reduced pressure. A solution of the residue in acetic acid–water (4 : 1 v/v; 20 cm^3) was heated, under

reflux, for 30 min. The products were evaporated under reduced pressure and then fractionated by short-column chromatography on silica gel; the appropriate fractions, which were eluted with chloroform–methanol (97 : 3 v/v) were evaporated under reduced pressure to give the *title compound* **10** (1.06 g, 15.8% overall yield for the 7 steps starting from uridine) (Found, in material crystallised from absolute ethanol: C, 53.5; H, 5.3; N, 7.3. $C_{17}H_{20}N_2O_6S$ requires C, 53.67; H, 5.30; N, 7.36%), mp 144–146 °C; δ_H [$(CD_3)_2SO$] 3.19 (1 H, dd, *J* 4.9 and 9.3), 3.63 (1 H, m), 3.72 (3 H, s), 3.73–3.83 (3 H, m), 4.01 (2 H, m), 5.27 (1 H, t, *J* 4.7), 5.58 (1 H, d, *J* 8.1), 5.65 (1 H, d, *J* 1.7), 5.92 (1 H, d, *J* 5.2), 6.84 (2 H, d, *J* 8.6), 7.24 (2 H, d, *J* 8.6), 8.02 (1 H, d, *J* 8.1), 11.29 (1 H, br s); δ_C [$(CD_3)_2SO$] 34.4, 45.3, 55.0, 59.4, 75.5, 84.5, 90.1, 100.9, 113.0, 130.1, 140.2, 150.4, 158.2, 163.2.

(b) A solution of 1-(2′,5′-di-*O*-trityl-β-D-xylofuranosyl)-uracil)[11] **15** (3.32 g, 4.5 mmol) and methanesulfonyl chloride (1.76 cm^3, 22.7 mmol) in dry pyridine (18 cm^3) was stirred at room temperature. After 24 h, the products were cooled to 0 °C (ice–water-bath) and saturated aq. sodium hydrogen carbonate (10 cm^3) was added, followed by dichloromethane (300 cm^3). The resulting mixture was extracted, first with saturated aq. sodium hydrogen carbonate (2 × 200 cm^3) and then with water (200 cm^3). The dried ($MgSO_4$) organic layer was evaporated under reduced pressure and the residue was co-evaporated with dry toluene (2 × 15 cm^3) to give the putative 3′-*O*-mesyl derivative **16** as a foam.

4-Methoxytoluene-α-thiol **7** (2.09 cm^3, 15 mmol) was added dropwise to a solution of sodium hydride (60% dispersion in mineral oil; 0.546 g, 13.65 mmol) in DMA (20 cm^3) at 0 °C (ice–water-bath). The cooling bath was then removed. The solution obtained was stirred at room temperature for 15 min and was then added to the above 3′-*O*-mesyl derivative **16**. The reactants were heated at 110 °C. After 2 h, the products were cooled and dichloromethane (200 cm^3) was added. The resulting solution was washed with saturated aq. sodium hydrogen carbonate (2 × 200 cm^3). The combined aqueous layers were back-extracted with dichloromethane (100 cm^3). The organic layers were combined, dried ($MgSO_4$), and evaporated under reduced pressure. The residue was heated with acetic acid–water (4 : 1 v/v; 30 cm^3), under reflux. After 30 min, the products were concentrated under reduced pressure and re-evaporated with cyclohexane (2 × 15 cm^3). The residue was fractionated by short-column chromatography on silica gel: the appropriate fractions, which were eluted with chloroform–methanol (99 : 1 to 98 : 2 v/v), were combined, and evaporated under reduced pressure to give the title compound **10** (0.265 g, 15.3% overall yield for the 3 steps), identical (^1H, ^{13}C NMR) to the material obtained in (a) above.

3′-*S*-(2,4-Dinitrophenylsulfanyl)-2′,5′-bis-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-3′-thiouridine 14

TFA (0.115 cm^3, 1.5 mmol) was added to a stirred solution of 3′-*S*-(4-methoxybenzyl)-3′-thiouridine **10** (0.190 g, 0.5 mmol) and 2,4-dinitrobenzenesulfenyl chloride **11** (0.234 g, 1.0 mmol) in dry dichloromethane (8 cm^3) at 0 °C (ice–water-bath). After 15 min, the products were evaporated under reduced pressure. A solution of the residue in chloroform–methanol (95 : 5 v/v; 10 cm^3) was pre-adsorbed on silica gel (3 g). The products were then fractionated by short-column chromatography on silica gel: elution of the column with chloroform–methanol (97 : 3 v/v) and concentration of the appropriate fractions gave 3′-*S*-(2,4-dinitrophenylsulfanyl)-3′-thiouridine **12** as a yellow solid (0.217 g); δ_H [$(CD_3)_2SO$] 3.56 (1 H, dd, *J* 5.3 and 9.0), 3.62 (1 H, m), 3.82 (1 H, m), 4.22 (1 H, m), 4.43 (1 H, m), 5.28 (1 H, m), 5.54 (1 H, d, *J* 8.1), 5.72 (1 H, d, *J* 2.0), 6.63 (1 H, d, *J* 5.4), 7.93 (1 H, d, *J* 8.2), 8.56 (1 H, d, *J* 9.0), 8.62 (1 H, dd, *J* 2.4 and 9.0), 8.90 (1 H, d, *J* 2.3), 11.34 (1 H, br s); δ_C [$(CD_3)_2SO$] 52.6, 59.7, 75.2, 84.2, 90.4, 101.2, 121.4, 128.2, 129.2, 140.3, 144.7, 145.0, 145.3, 150.4, 163.2.

The above material **12** (0.224 g) and 1-(2-fluorophenyl)-4-methoxy-1,2,3,6-tetrahydropyridine[13] **13** (0.71 g, 3.4 mmol) were dissolved in dry acetonitrile (6 cm^3) and the resulting solution was evaporated under reduced pressure. After this process had been repeated, the residue was dissolved in dichloromethane (9 cm^3), and TFA (0.29 cm^3, 3.8 mmol) was added. The reaction solution was stirred at room temperature. After 17 h, triethylamine (1.05 cm^3, 7.5 mmol) was added and the products were evaporated under reduced pressure. The residue was dissolved in chloroform (50 cm^3) and the resulting solution was washed with saturated aq. sodium hydrogen carbonate (2 × 25 cm^3). The combined aqueous layers were back-extracted with chloroform (25 cm^3). The combined organic layers were dried ($MgSO_4$), and evaporated under reduced pressure. The residue was fractionated by short-column chromatography on silica gel: the appropriate fractions, which were eluted with chloroform–methanol (95.5 : 0.5 to 99 : 1 v/v), were combined and evaporated under reduced pressure to give the *title compound* **14** as a yellow foam [0.32 g; ≈70% overall yield for the two steps based on 3′-*S*-(4-methoxybenzyl)-3′-thiouridine **10** as starting material]; δ_H [$(CD_3)_2SO$] 1.73 (4 H, m), 1.86 (1 H, m), 2.00 (3 H, m), 2.75–2.95 (6 H, m), 3.05 (3 H, s), 3.15 (5 H, m), 3.51 (1 H, dd, *J* 4.1 and 11.0), 3.62 (1 H, m), 3.93 (1 H, m), 4.45 (1 H, m), 4.98 (1 H, t, *J* 6.0), 5.77 (1 H, d, *J* 8.1), 6.07 (1 H, d, *J* 5.3), 6.8–7.2 (8 H, m), 7.89 (1 H, d, *J* 8.1), 8.48 (1 H, d, *J* 9.0), 8.54 (1 H, dd, *J* 2.4 and 9.0), 8.82 (1 H, d, *J* 2.3), 11.49 (1 H, br s); δ_C [$(CD_3)_2SO$] 32.5, 32.6, 32.8, 33.5, 47.3, 47.4, 47.5, 47.7, 48.0, 52.5, 60.2, 72.9, 79.2, 82.3, 87.2, 98.2, 100.5, 102.5, 140.8, 150.7, 162.9 and signals assignable to the resonances of the 18 aryl carbon atoms.

Triethylammonium salt of 3-*N*-benzoyl-2′,3′-di-*O*-benzoyl-uridine 5′-H-phosphonate 18

Uridine **4** (5.00 g, 20.5 mmol), chlorotriphenylmethane (6.29 g, 22.6 mmol) and pyridine (60 cm^3) were stirred together in an atmosphere of argon at 100 °C. After 1.5 h, the products were cooled to 0 °C (ice–water-bath) and benzoyl chloride (21.43 cm^3, 0.184 mol) was added. The reactants were then allowed to warm up to room temperature. After 14 h, the stirred products were cooled to 0 °C and water (20 cm^3) was added slowly. After a further period of 10 min, solid sodium hydrogen carbonate (20 g) was added very carefully in portions. After 15 min, the products were concentrated under reduced pressure and the residue was partitioned between chloroform (400 cm^3) and saturated aq. sodium hydrogen carbonate (300 cm^3). The solid precipitate was removed by filtration and the layers were separated. The organic layer was back-extracted with chloroform (2 × 150 cm^3). The combined organic layers were dried ($MgSO_4$), and evaporated under reduced pressure. After the residue had been coevaporated with toluene under reduced pressure, it was dissolved in acetic acid–water (4 : 1 v/v; 75 cm^3) and the solution was heated under reflux. After 1.5 h, the products were concentrated under reduced pressure and the residue was coevaporated with cyclohexane (2 × 50 cm^3). The residual material was fractionated by short-column chromatography on silica gel: the appropriate fractions, which were eluted with chloroform–methanol (99 : 1 v/v), were combined, and evaporated under reduced pressure. Crystallisation of the residual glass from absolute ethanol gave 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine **17** as colourless needles (5.51 g, 48.3%), mp 191–193 °C (lit.[14] 191–193 °C); δ_H [$(CD_3)_2SO$] 3.83 (2 H, m), 4.53 (1 H, m), 5.63 (1 H, m), 5.79 (2 H, m), 6.12 (1 H, d, *J* 8.2), 6.30 (2 H, d, *J* 5.3), 7.40 (2 H, m), 7.45–7.7 (6 H, m), 7.77 (3 H, m), 7.79 (4 H, m), 8.31 (1 H, d, *J* 8.2).

Dry triethylamine (5.42 cm^3, 39.0 mmol) and phosphorus trichloride (1.05 cm^3, 12.0 mmol) were added to a stirred solution of 1*H*-1,2,4-triazole (2.48 g, 36.0 mmol; recrystallised from dry acetonitrile) in dry THF (72 cm^3) at −35 °C (methanol–solid CO_2-bath). After 15 min, a solution of 3-*N*-benzoyl-2′,3′-

di-*O*-benzoyluridine **17** (1.67 g, 3.00 mmol) in dry THF (60 cm^3) was added. The stirred reactants were maintained at −35 °C. After a further period of 30 min, triethylamine–water (1:1 v/v; 20 cm^3) was added. The products were allowed to warm up to room temperature and were then concentrated under reduced pressure. The residue was partitioned between chloroform (200 cm^3) and 0.5 mol dm^{-3} triethylammonium hydrogen carbonate (pH 7.5; 2 × 100 cm^3). The organic layer was dried ($MgSO_4$), and evaporated under reduced pressure. The residue was fractionated by short-column chromatography on silica gel: the appropriate fractions, which were eluted with chloroform–methanol (95:5 v/v), were combined, and evaporated under reduced pressure. A solution of the residue in chloroform (15 cm^3) was added dropwise to stirred petroleum spirit (30–40 °C; 400 cm^3) to give triethylammonium 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine 5′-H-phosphonate **18** as a colourless solid (1.95 g, ≈90%, based on 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine **17**); t_R 8.02 min (programme 1); δ_H [$(CD_3)_2SO$–D_2O] 1.15 (6 H, t, *J* 7.3), 3.03 (4 H, q, *J* 7.3), 4.11 (2 H, m), 4.63 (1 H, m), 5.77 (2 H, m), 5.90 (0.5 H, s), 6.14 (1 H, d, *J* 8.2), 6.28 (1 H, d, *J* 5.5), 7.36 (2 H, t, *J* 7.8), 7.4–7.7 (6.5 H, m), 7.73 (3 H, m), 7.94 (4 H, m), 8.34 (1 H, d, *J* 8.3); δ_P 2.6 (d, $J_{P,H}$ 602.8).

Triethylammonium salt of 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] 3

3′-*S*-[(2,4-Dinitrophenylsulfanyl]-2′,5′-bis-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl]-3′-thiouridine **14** (0.087 g, ≈0.1 mmol) and the triethylammonium salt of 3-*N*-benzoyl-2′,3′-di-*O*-benzoyluridine 5′-H-phosphonate **18** (0.144 g, ≈0.2 mmol) were dissolved in dry acetonitrile (10 cm^3) and the resulting solution was evaporated under reduced pressure. The residue was then dissolved in dry dichloromethane (4 cm^3), and dry triethylamine (0.167 cm^3, 1.2 mmol) and chlorotrimethylsilane (0.13 cm^3, 1.0 mmol) were added. The reactants were then stirred at room temperature. After 17 h, the products were poured into 0.2 mol dm^{-3} aq. triethylammonium hydrogen carbonate (pH 7.5; 100 cm^3) and the resulting mixture was extracted with chloroform (3 × 100 cm^3). The combined organic extracts were dried ($MgSO_4$), and evaporated under reduced pressure. The residue, compound **19**, was dissolved in 2 mol dm^{-3} methanolic ammonia (5 cm^3) and the resulting solution was stirred at room temperature. After 17 h, the products were evaporated under reduced pressure. The residue was fractionated on a column of Merck silanised silica gel: the appropriate fractions, which were eluted with acetonitrile–water (15:85 v/v), were combined, and evaporated under reduced pressure. The residue was then rechromatographed on a column (17 cm × 2 cm diameter) of DEAE Sephadex A-25, which was eluted with a linear gradient (0.0 to 0.50 mol dm^{-3} over 600 cm^3) of triethylammonium hydrogen carbonate buffer (pH 7.5). The appropriate fractions, which were eluted with an average buffer concentration of 0.34 mol dm^{-3}, were combined, and concentrated under reduced pressure. The residue was coevaporated with ethanol (2 × 10 cm^3) under reduced pressure and then dissolved in chloroform (2 cm^3). When this solution was added, with stirring, to petroleum spirit (30–40 °C, 100 cm^3), a colourless solid precipitate (0.070 g) was obtained. This material (0.035 g) was dissolved in acetic acid–water (2:98 v/v; 5 cm^3) and the solution was stirred at room temperature. After 17 h, the products were evaporated under reduced pressure. The residue was coevaporated with cyclohexane (3 × 5 cm^3); it was then fractionated on a column (17 cm × 2 cm diameter) of DEAE Sephadex A-25, which was eluted as above. The appropriate fractions, which were eluted with an average buffer concentration of 0.22 mol dm^{-3}, were combined, and evaporated under reduced pressure. The residue was coevaporated under reduced pressure with ethanol (2 × 5 cm^3) to give the triethylammonium salt of 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] **3** as a colourless solid (387 A_{260} units); t_R 7.70 min (programme 2) (Fig. 1b); δ_H (D_2O) 1.24 (9 H, m), 3.16 (6 H, m), 3.35 (1 H, dt, *J* 4.7 and 11.9), 3.90 (1 H, dd, *J* 3.1 and 13.6), 4.04 (1 H, d, *J* 13.8), 4.11 (2 H, m), 4.24 (3 H, m), 4.35 (1 H, dd, *J* 4.9 and 11.7), 4.42 (1 H, d, *J* 4.6), 5.68 (1 H, d, *J* 7.7), 5.70 (1 H, d, *J* 8.1), 5.72 (1 H, d, *J* 8.3), 5.82 (1 H, d, *J* 2.1), 7.98 (1 H, d, *J* 8.1), 8.02 (1 H, d, *J* 8.2); δ_P (D_2O) 18.7 (Fig. 1a).

Triethylammonium salt of 3′-thiouridine 3′-phosphorothioate 20

3′-*S*-(2,4-Dinitrophenylsulfanyl)-3′-thiouridine **12** (0.046 g, ≈0.1 mmol), prepared as above by the action of 2,4-dinitrobenzenesulfenyl chloride and TFA on 3′-*S*-(4-methoxybenzyl)-3′-thiouridine **10**, was dissolved in dry acetonitrile (5 cm^3) and the resulting solution was evaporated under reduced pressure. Triethylamine (0.076 cm^3, 0.55 mmol) and chlorotrimethylsilane (0.064 cm^3, 0.5 mmol) were added to a stirred suspension of the residue in dichloromethane (4 cm^3) at room temperature. After 15 min, tris(trimethylsilyl) phosphite (0.05 cm^3, 0.15 mmol) was added. After a further period of 30 min, the products were evaporated under reduced pressure. The residue was dissolved in 0.2 mol dm^{-3} aq. triethylammonium hydrogen carbonate (pH 7.5, 10 cm^3) and the resulting solution was extracted with dichloromethane (10 cm^3). The aqueous layer was concentrated to small volume under reduced pressure and was then applied to a column (17 cm × 2 cm diameter) of DEAE Sephadex A-25, which was eluted with a linear gradient (0.0 to 0.50 mol dm^{-3} over 600 cm^3) of triethylammonium hydrogen carbonate buffer (pH 7.5). The appropriate fractions, which were eluted with an average buffer concentration of 0.34 mol dm^{-3}, were combined and lyophilised to give the triethylammonium salt of 3′-thiouridine 3′-phosphorothioate **20** as a colourless solid [507 A_{260} units; t_R 6.24 min (programme 2)] (Fig. 2a); δ_H (D_2O) includes the following signals: 3.55 (1 H, dt, *J* 4.8 and 11.2), 3.99 (1 H, dd, *J* 3.2 and 13.5), 4.05 (1 H, dd, *J* 2.1 and 13.4), 4.13 (1 H, m), 4.49 (1 H, d, *J* 4.8), 5.86 (1 H, s), 5.88 (1 H, d, *J* 8.2), 8.08 (1 H, d, *J* 8.1); δ_P (D_2O) 16.4 (d, $J_{P,H}$ 11.2).

Triethylammonium salt of 3′-thiouridine 2′,3′-cyclic phosphorothioate 21

Triethylammonium 3′-thiouridine 3′-phosphorothioate **20** (210 A_{260} units), tributylamine (0.12 cm^3, 0.5 mmol), ethyl chloroformate (0.019 cm^3, 0.2 mmol) and water (0.5 cm^3) were vigorously stirred together at room temperature. After 40 min, methanol (0.5 cm^3) was added and the products were applied to a column (17 cm × 2 cm diameter) of DEAE Sephadex A-25. The column was eluted with a linear gradient (0.0 to 0.50 mol dm^{-3} over 600 cm^3) of triethylammonium hydrogen carbonate buffer (pH 7.5). Appropriate fractions, which were eluted with an average buffer concentration of 0.22 mol dm^{-3}, were combined and lyophilised. The residual solid was dissolved in water (2 cm^3) and the solution was applied to a column (30 × 2 cm diameter) of Merck silanised silica gel. The product-containing fractions, which were eluted with water, were combined and lyophilised to give the triethylammonium salt of 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** as a colourless solid (160 A_{260} units); t_R 4.69 min (programme 2) (Fig. 2b); δ_H (D_2O) includes the following signals: 3.76 (1 H, dd, *J* 4.2 and 13.0), 3.94 (1 H, dd, *J* 2.2 and 12.9), 4.1–4.25 (2 H, m), 4.90 (1 H, m), 5.84 (1 H, d, *J* 8.1), 5.98 (1 H, s), 7.79 (1 H, d, *J* 8.1); δ_P (D_2O) 37.0 (d, $J_{P,H}$ 13.5).

Hydrolysis of 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] 3 in 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.06) at 50 °C

Substrate [(Us)pU] **3** (1 A_{260} unit) was dissolved in sodium glycinate buffer (0.15 cm^3) and the resulting solution was heated at 50 °C. After appropriate intervals of time, aliquots (10 mm^3)

of reaction solution were removed, and analysed by HPLC (programme 2). A straight line was obtained by plotting ln [%(Us)pU remaining] against time. The half-life ($t_{1/2}$) of hydrolysis was found to be 25 min. A typical HPLC profile (Fig. 3a) obtained after 45 min revealed unchanged (Us)pU **3** (t_R 7.76 min, 25.3% of total absorbance), uridine **4** (t_R 3.37 min, 37.0% of total absorbance), 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** (t_R 4.83 min, 14.0% of total absorbance) and 3′-thiouridine 3′-phosphorothioate **20** (t_R 6.27 min, 22.9% of total absorbance). In a separate experiment, the substrate [(Us)pU] (30 A_{260} units) was dissolved in the same sodium glycinate buffer (3.0 cm^3) and the resulting solution was heated at 50 °C. After 30 min, acetic acid was added until the pH dropped to 4.0. The products were then evaporated under reduced pressure. The ^{31}P NMR spectrum of the residue (in D_2O) (Fig. 4a) revealed the following resonance signals: δ 37.2 (s, ≈20%, assigned to 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**), 18.7 [s, ≈70%, assigned to unchanged (Us)pU **3**] and 16.3 (s, ≈10%, assigned to 3′-thiouridine 3′-phosphorothioate **20**). The half-life of hydrolysis of uridylyl-(3′→5′)-uridine (UpU) **1a** in the same sodium glycinate buffer solution was found to be 80–90 h at 50 °C.

Action of glacial acetic acid on 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] 3 at 30 °C

A freshly prepared solution of (Us)pU **3** (1 A_{260} unit) in glacial acetic acid (0.15 cm^3) was maintained at 30 °C. After appropriate intervals of time, aliquots (10 mm^3) of the solution were removed, rapidly evaporated under reduced pressure (oil-pump), re-dissolved in water (15 mm^3) and analysed by HPLC (programme 2). The half-life of decomposition of (Us)pU **3** was found to be *ca.* 4 min. A typical HPLC profile, after a reaction time of 10 min (Fig. 3b), revealed uridine **4** (t_R 3.3 min, 38.3% of total absorbance), 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** (t_R 4.71 min, 36.2% of total absorbance), 3′-thiouridine 3′-phosphorothioate **20** (t_R 6.19 min, 4.6% of total absorbance), an unidentified product that might be 3′-thiouridylyl-(2′→5′)-uridine **22** (t_R 7.51 min, 2.3% of total absorbance) and unchanged (Us)pU **3** (t_R7.67 min, 18.6% of total absorbance). In a separate experiment, the substrate [(Us)pU] **3** (10 A_{260} units) was dissolved in glacial acetic acid (0.50 cm^3) and the resulting solution was maintained at 30 °C. After 30 min, the products were evaporated under reduced pressure (oil-pump). The ^{31}P NMR spectrum (D_2O) (Fig. 4b) of the residue revealed the following resonance signals: δ 37.2 (s, ≈81%, assigned to 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**), 18.7 [s, *ca.* 11%, assigned to (Us)pU **3**], 16.8 (s, ≈5%, assigned to 3′-thiouridine 3′-phosphorothioate **20**) and −0.44 [s, ≈3%, assigned tentatively to 3′-thiouridylyl-(2′→5′)-uridine **22**].

Action of glacial acetic acid on uridylyl-(3′→5′)-uridine [UpU] 1a at 30 °C

The above experiment was repeated with UpU **1a** (1 A_{260} unit). Aliquots were removed and evaporated as above, and then analysed by HPLC (programme 3). After 10 min, the absorbance percentages relating to remaining substrate **1a** (t_R 9.27 min), uridylyl-(2′→5′)-uridine **23** (t_R 7.71 min), uridine 2′,3′-cyclic phosphate **24** (t_R 3.53 min) and uridine **4** (t_R 3.29 min) were 87.8, 4.5, 3.7 and 4.0. After 37 min (Fig. 3c), the absorbance percentages relating to the remaining substrate **1a**, uridylyl-(2′→5′)-uridine **23**, uridine 2′(3′)-phosphates **25** and **26** (t_R 4.99, 5.17), uridine 2′,3′-cyclic phosphate **24** and uridine **4** were 63.9, 12.8, 1.5, 10.0 and 11.8. After 60 min, the absorbance percentages relating to remaining substrate **1b**, uridylyl-(2′→5′)-uridine **23**, uridine 2′(3′)-phosphates **25** and **26**, uridine 2′,3′-cyclic phosphate **24** and uridine **4** were 49.8, 15.7, 4.6, 12.3 and 17.6.

Hydrolysis of 3′-thiouridine 2′,3′-cyclic phosphorothioate 21 in 0.05 mol dm^{-3} sodium glycinate buffer

Substrate **21** (1 A_{260} unit) was dissolved in 0.05 mol dm^{-3} sodium glycinate buffer (pH 9.87; 0.15 cm^3) and the resulting solution was maintained at 30 °C. After appropriate intervals of time, aliquots (10 mm^3) of reaction solution were removed and analysed by HPLC (programme 2). A straight line was obtained by plotting ln (% substrate **21** remaining) against time. The half-life ($t_{1/2}$) of hydrolysis was found to be 164 min. A typical HPLC profile, obtained after 131 min, revealed unchanged substrate **21** (t_R 4.82 min, 54% of total absorbance) and 3′-thiouridine 3′-phosphorothioate **20** (t_R 6.27 min, 46% of total absorbance). In a separate experiment, the substrate (10 A_{260} units) was dissolved in a mixture of 0.05 mol dm^{-3} sodium glycinate buffer (pH 10.6; 0.30 cm^3) and D_2O (0.30 cm^3 at room temperature. The resulting solution was maintained at 21 °C in an NMR tube. After ≈7.4 h, the ^{31}P NMR spectrum revealed two resonance signals: δ 37.3 (s, ≈40%, assigned to 3′-thiouridine 2′,3′-cyclic phosphorothioate **21**) and 16.0 (s, ≈60%, assigned to 3′-thiouridine 3′- phosphorothioate **20**).

Action of hydrolytic enzymes on 3′-thiouridylyl-(3′→5′)-uridine [(Us)pU] 3

(a) Ribonuclease A. A solution of ribonuclease A (10 μg) in 0.1 mol dm^{-3} Tris hydrochloride buffer (pH 7.5; 10 mm^3) was added to a solution of (Us)pU **3** (1 A_{260} unit) in the same buffer solution (0.1 cm^3). The resulting solution was maintained at 30 °C. After 26 min, HPLC analysis (programme 2) revealed substrate **3** (t_R 7.6 min, 15.7% of total absorbance), 3′-thiouridine 3′-phosphorothioate **20** (t_R 6.13 min, 28.3% of total absorbance), 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** (t_R 4.60 min, 15.0% of total absorbance) and uridine **4** (t_R 3.19 min, 41.0% of total absorbance). After 146 min, HPLC analysis revealed substrate **3** (0.3% of total absorbance), 3′-thiouridine 3′-phosphorothioate **20** (50.4% of total absorbance) and uridine (49.3% of total absorbance). Under the same reaction conditions, 3′-thiouridine 2′,3′-cyclic phosphorothioate **21** underwent 30% digestion (to 3′-thiouridine 3′-phosphorothioate **20**) by ribonuclease A in 5 min.

(b) *Crotalus adamanteus* snake venom phosphodiesterase. A stock solution of enzyme in 0.1 mol dm^{-3} Tris hydrochloride buffer (pH 7.5; 10 mm^3) was added to a solution of (Us)pU **3** (1 A_{260} unit) in 0.1 mol dm^{-3} Tris hydrochloride buffer (pH 7.5; 0.01 mol dm^{-3} with respect to magnesium chloride; 0.10 cm^3). The reaction solution was maintained at 30 °C. After 4 h, HPLC analysis (programme 4) revealed that the substrate accounted for only 9% of the total absorbance at 260 nm.

(c) Calf spleen phosphodiesterase. A stock solution of enzyme in 0.1 mol dm^{-3} Tris hydrochloride buffer (pH 7.5; 10 mm^3) was added to a solution of (Us)pU **3** (1 A_{260} unit) in 0.1 mol dm^{-3} Tris hydrochloride buffer (pH 7.5; 0.002 mol dm^{-3} with respect to EDTA and containing 0.05% Tween 80; 0.10 cm^3). The solution was maintained at 30 °C. The substrate remained completely undigested after 24 h. Under the same conditions, uridylyl-(3′→5′)-uridine (UpU) **1a** was quantitatively digested to uridine 3′-phosphate and uridine **4**.

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