Synthesis and Properties of Diuridine Phosphate Analogues Containing Thio and Amino Modifications

James B. Thomson,† Bhisma K. Patel,† Victor Jiménez,‡ Klaus Eckart, and Fritz Eckstein*

Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Str.3, D-37075 Göttingen, Germany

Received April 30, 1996⊗

Several analogues of diuridine phosphate (UpU) were synthesized in order to investigate why replacing the 2′-hydroxyl with a 2′-amino group prevents hydrolysis. These analogues were designed to investigate what influence the 2′-substituent and 5′-leaving group have upon the rate of hydrolysis. All the analogues were considerably more labile than UpU toward acid−base-catalyzed hydrolysis. In the pH region from 6 to 9, the rate of hydrolysis of uridylyl (3′−5′) 5′-thio-5′-deoxyuridine (UpsU) hydrolysis rose, in a log linear fashion, from a value of 5×10^{-6} s^{-1} at pH 6 to 3200×10^{-6} s^{-1} at pH 9, indicating that attack on the phosphorus by the 2′-oxo anion is rate-limiting in the hydrolysis mechanism. In contrast, the rate of uridylyl (3′−5′) 5′-amino-5′-deoxyuridine (UpnU) hydrolysis fell from a value of 1802×10^{-6} s^{-1} at pH 5 to 140×10^{-6} s^{-1} at pH 7.5, where it remained constant up to pH 11.5, thus indicating an acid-catalyzed reaction. The analogue 2′-amino-2′-deoxyuridylyl (3′−5′) 5′-thio-5′-deoxyuridine (amUpsU) was readily hydrolyzed above pH 7, in contrast to the hydrolytic stability of amUpT, with rates between 85×10^{-6} s^{-1} and 138×10^{-6} s^{-1}. The hydrolysis of 2′-amino-2′-deoxyuridylyl (3′−5′) 5′-amino-5′-deoxythymidine (amUpnT) rose from 17×10^{-6} s^{-1} at pH 11.5 to $11\,685 \times 10^{-6}$ s^{-1} at pH 7.0, indicating an acid-catalyzed reaction, where protonation of the 5′-amine is rate limiting. The cleavage rates of UpsU, UpnU, and amUpsU were accelerated in the presence of Mg^{2+}, Zn^{2+}, and Cd^{2+} ions, but a correlation with interaction between metal ion and leaving group could only be demonstrated for amUpsU. UpsU and UpnU are also substrates for RNase A with UpsU having similar Michaelis−Menten parameters to UpU. In contrast, UpnU is more rapidly degraded with an approximate 35-fold increase in catalytic efficiency, which is reflected purely in an increase in the value of k_{cat}.

Introduction

The interest in RNA-catalyzed reactions has stimulated structural[1−6] and chemical modification[7] studies of RNA in order to better understand both the structure−function relationships and the mechanisms of such reactions. One such catalytic RNA is the hammerhead ribozyme, which most efficiently cleaves its substrate after a GUC sequence to yield a 2′,3′-cyclic phosphate and a 5′-hydroxyl termini.[8] We have recently shown that replacement of the 2′-hydroxy at the cleavage site by an amino group prevents this cleavage[9] and such a modification also stabilizes the RNA toward alkaline and RNase A-mediated hydrolysis.[9−12] Nucleotides bearing this modification have been utilized for *in vitro* selection protocols to obtain nuclease-resistant RNA aptamers for therapeutic applications.[13−15] In order to understand this hydrolytic stability we have undertaken a study to determine whether cleavage could be obtained with the 2′-amino group by variation of the leaving group. We report here a comparative study of the hydrolysis of UpU with that of UpsU, amUpsU, UpnU, and amUpnT (Figure 1) as a function of pH, divalent metal ions, and RNaseA.

Results

The four modified dinucleotides were synthesized by the reaction schemes outlined in Schemes 1 and 2. Additionally, TpsT and TpnT were prepared by similar routes for use as control compounds containing no 2′-nucleophile.

Hydrolysis of Dinucleotides. In order to establish whether the buffer could enhance the hydrolysis rate of the dimers, the dependence of k_{obs} on the buffer concentration was established for UpsU and UpnU at pH 7.5 using HEPES buffer. Their rate of hydrolysis demonstrated a slight positive linear dependence upon the buffer concentration, as a result of the weak general base properties of HEPES. Extrapolating both plots to zero

† Both authors have contributed equally to this publication.

‡ Genetic Engineering and Biotechnology Center, PO Box 6162 Havana, Cuba.

* Author to whom correspondance should be addressed. Tel: +49-551-3899-274. Fax: +49-551-3899-388. E-mail: eckstein@exmed1.dnet.gwdg.de.

⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1996.

(1) Pley, H. W.; Flaherty, K. M.; McKay, D. B. *Nature* **1994**, *372*, 68−74.

(2) Scott, W. G.; Finch, J. T.; Klug, A. *Cell (Cambridge, Mass.)* **1995**, *81*, 991−1002.

(3) Tuschl, T.; Gohlke, C.; Jovin, T. M.; Westhof, E.; Eckstein, F. *Science* **1994**, *266*, 785−788.

(4) Tanner, N. K.; Schaff, S.; Thill, G.; Petit-Koskas, E.; Crain-Denoyelle, A.-M.; Westhof, E. *Curr. Biol.* **1994**, *4*, 488−498.

(5) Westhof, E.; Altman, S. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5133−5137.

(6) Cech, T. R.; Damberger, S. H.; Gutell, R. R. *Nature Struct. Biol.* **1994**, *1*, 273−280.

(7) Grasby, J. A.; Gait, M. J. *Biochimie* **1994**, *76*, 1223−1234.

(8) Uhlenbeck, O. C. *Nature* **1987**, *328*, 596−600.

(9) Pieken, W. A.; Olsen, D. B.; Benseler, F.; Aurup, H.; Eckstein, F. *Science* **1991**, *253*, 314−317.

(10) Hobbs, J.; Sternbach, H.; Sprinzl, M.; Eckstein, F. *Biochemistry* **1973**, *12*, 5138−4145.

(11) Heidenreich, O.; Benseler, F.; Fahrenholz, A.; Eckstein, F. *J. Biol. Chem.* **1994**, *269*, 2131−2138.

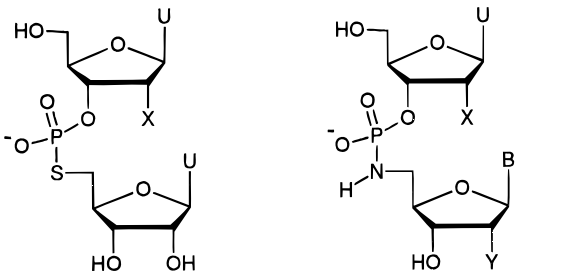
(12) Sigurdsson, S. T.; Tuschl, T.; Eckstein, F. *RNA* **1995**, *1*, 575−583.

(13) Jellinek, D.; Green, S. L.; Bell, C.; Lynott, C. K.; Gill, N.; Vargeese, C.; Kirschenheuter, G.; McGee, D. P. C.; Abesinghe, P.; Pieken, W. A.; Shapiro, R.; Rifkin, D. B.; Moscatelli, D.; Janjic, N. *Biochemistry* **1995**, *34*, 11363−11372.

(14) Eaton, B. E.; Pieken, W. A. *Annu. Rev. Biochem.* **1995**, *64*, 837−863.

(15) Gold, L.; Polisky, B.; Uhlenbeck, O.; Yarus, M. *Annu. Rev. Biochem.* **1995**, *64*, 763−797.

S0022-3263(96)00795-5 CCC: $12.00

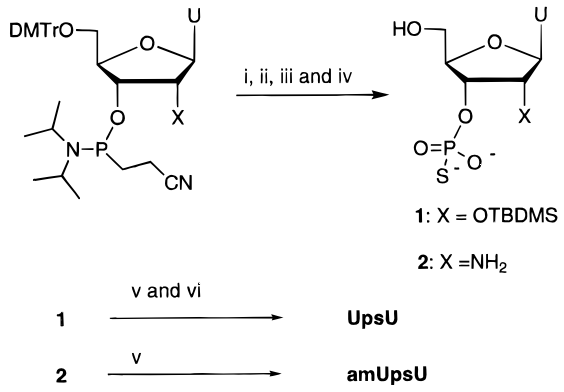


UpsU: X = OH
amUpsU: X = NH_2

UpnU: X, Y = OH, B = U
amUpnT: X = NH_2, Y = H, B = T

Figure 1. Modified uridine dimers investigated in this study. U = uridine and T = thymidine.

Scheme 1.[a] **Synthesis of UpsU and amUpsU**



[a] (i) 3-Hydroxypropionitrile, tetrazole; (ii) 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent); (iii) ammonia; (iv) 80% AcOH; and (v) 5′-iodo-5′-deoxyuridine; (vi) triethylammonium trihydrogen fluoride.

buffer strength gave k_{obs} values of 92 and 94 $\times$ 10^{-6} s^{-1}, for UpsU and UpnU, respectively. This is very close to the values of 97 and 140 $\times$ 10^{-6} s^{-1} obtained in 100 mM HEPES. This is also supported by the agreement between our data and that obtained in a study[16] where TRIS was used as the buffering agent. For the purposes of this study, the buffer effect was therefore ignored.

The rate of hydrolysis of the dinucleotides, k_{obs}, was carried out in the pH range from 5 to 11.5 (Figure 2). The hydrolytic cleavage of UpU was extremely slow under the assay conditions, and degradation to uridine and 2′,3′-cUMP was only observed at pH 11.5 (k_{obs} = 14 $\times$ 10^{-6} s^{-1}). In contrast, UpsU, amUpsU, UpnU, and amUpnT were readily hydrolyzed but gave markedly different pH profiles as discussed below.

The plot of log k_{obs} versus pH, for UpsU (Figure 2) increases linearly, with increasing pH in the region from pH 6 to 9, with a gradient of 0.9 indicating that hydrolysis is base-catalyzed. This is consistent with the observations made by Liu and Reese,[16] also with UpsU. The hydrolysis of UpsU proceeded exclusively to 2′,3′-cUMP, 5′-thio-5′-deoxyuridine (**10**), and its disulfide (**11**) (Table 1). In contrast, UpnU hydrolysis is log linear in the pH range from 5 to 6, with a slope of −0.8, which indicates acid catalysis in this region. By pH 7.5 the hydrolysis rate has plateaued to a value of 140 $\times$ 10^{-6} s^{-1}, where it

(16) Liu, X.; Reese, C. B. *Tetrahedron Lett.* **1995**, *36*, 3413−3416.

Table 1. ^{31}P NMR Chemical Shifts and HPLC Retention Times of Uridine Dinucleotides and Their Hydrolysis Products

dimer and degradation products	δ (ppm)	t_R[a] (min)
UpU	0 (q, J_1 = 2.9 Hz, J_2 = 4.6 Hz)	11
2′(3′)-UMP	4 (d, J = 7.8 Hz)	8.5
2′,3′-cUMP	20 (q, J_1 = 6.2 Hz, J_2 = 12.1 Hz)	6.6
uridine		7.2
UpsU	20.4	13
10		13.6
11		21
UpnU	9.3	11.4
5′-aminoU		1.6
amUpnT	9.3	10.7
amUpsU	20.2 (q, J_1 = 11.2 Hz, J_2 = 21.9 Hz)	12.6
12	28 (m)	6.1
13	7.5 (d, J = 6.2 Hz)	7.2
9	4.2 (d, J = 8.4 Hz)	5.3

[a] All HPLC analysis were performed using buffer system 1 and gradient 1.

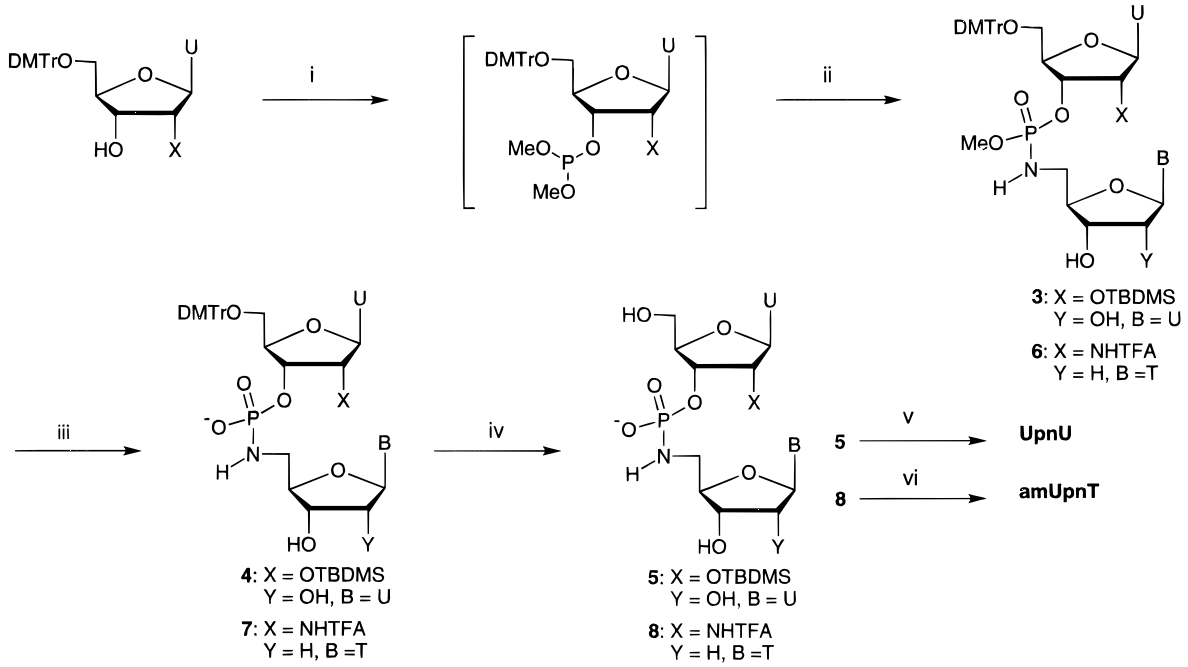
remains constant up to pH 11.5. UpnU is exclusivly hydrolyzed to 2′,3′-cUMP and 5′-amino-5′-deoxyuridine (Table 1).

The hydrolysis of the dinucleotide amUpsU is pH independent between pH 7 and 11.5, maintaining a steady rate of 85 −138 $\times$ 10^{-6} s^{-1} (Figure 2). However, below pH 7 the log rate of hydrolysis decreases, with a slope of 0.4, which indicates inactivation by acid and is consistent with protonation of the 2′-amine (pK_a = 6.2)[17,18] to give the non-nucleophilic ammonium species. Hydrolysis of amUpsU follows two distinct pathways, with the final hydrolysis products being dependent upon the pH of the reaction mixture (Scheme 3). Hydrolysis at pH 6 yields uridine 3′-monophosphate (**9**), 5′-thio-5′-deoxyuridine (**10**) and its dimer **11**. The cyclic phosphoramidate intermediate **12** was not observed, presumably a result of its rapid degradation to **9**. Cleavage of the cyclic P−N bond is clearly more facile than that observed for an acyclic P−N bond, such as in TpnT, where it is stable to hydrolysis down to pH 5. This is consistent with the observation that five-membered cyclic phosphates are hydrolyzed at a faster rate than their acyclic counterparts.[19] On following the course of hydrolysis of amUpsU at pH 10, compounds **12**, **10**, and **11** can be detected first. After 2 h incubation **13** appears, which reaches the same concentration as **12** after 10 h of incubation, after which time amUpsU is completely hydrolyzed. After incubation for 12 h, 2′-amino-2′-deoxyuridine (**14**) and inorganic phosphate (**15**) can be detected. Thus, under alkaline conditions, where the nitrogen atom of the cyclic phosphoramidate **12** is not protonated, it is more stable and degrades exclusively with P−O bond cleavage. There is a precedence for both P−O and P−N cleavage in five-membered 1,3,2,-oxazaphospholidin-2-ones, the level of which is very dependent upon the nature of both the phosphorus and nitrogen substituents.[20]

The hydrolysis of dinucleotide amUpnT increases log linearly with decreasing pH between pH 11.5 and 7, with a gradient of −0.7, indicating that the reaction is acid-catalyzed. Hydrolysis proceeded to yield 5′-amino-5′-

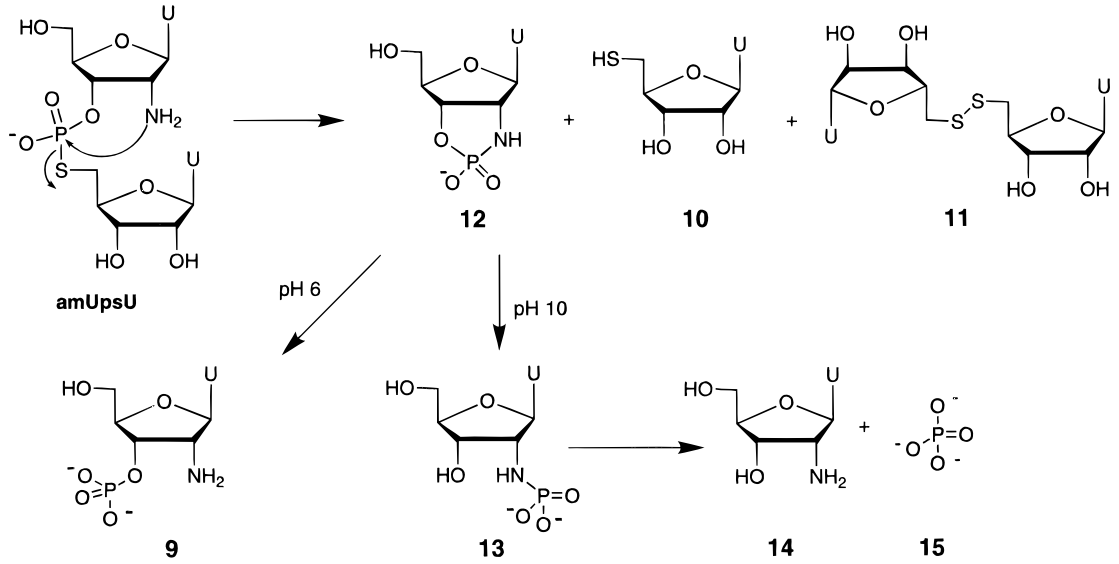
(17) Aurup, H.; Tuschl, T.; Benseler, F.; Ludwig, J.; Eckstein, F. *Nucleic Acids Res.* **1994**, *22*, 20−24.
(18) Miller, P. S.; Bhan, P.; Kan, L.-S. *Nucleosides Nucleotides* **1993**, *12*, 785−792.
(19) Kumamoto, J.; Cox, J. R.; Westheimer, F. H. *J. Am. Chem. Soc.* **1956**, *78*, 4858−4860.
(20) Hall, C. R.; Inch, T. D. *Tetrahedron* **1980**, *36*, 2059−2095.

Scheme 2.[a] Synthesis of UpnU and amUpnT



[a] (i) (*N*,*N*′-diisopropylamino)dimethoxyphosphane, tetrazole; (ii) 5′-azido-5′-deoxynucleoside, LiCl, pyridine; (iii) thiophenol, triethylamine; (iv) 80% AcOH; (v) tetrabutylammonium fluoride, and (vi) Na_2CO_3/MeOH/H_2O.

Scheme 3. Hydrolysis Products of amUpsU at pH 6 and 10



deoxythymidine (Table 1) and compound **12**, which subsequently degraded as indicated in Scheme 3.

The dinucleotides TpsT, TpnT, and amUpT[9] were completely stable toward hydrolysis under these conditions, emphasizing the requirement of an internal 2′-hydroxyl nucleophile for hydrolysis.

The reaction products of dinucleotide hydrolysis reactions were identified by comparison with authentic samples on HPLC and by ^{31}P NMR spectroscopy (Table 1). The hydrolysis products **9**, **10**, **11**, **14**, and **15** were identified by HPLC and, where possible, ^{31}P NMR spectroscopic (pH 10) comparison with authentic samples. To the best of our knowledge there is no data available for cyclic 1,2,3-oxazaphospholidin-2-ones, such as **12**, with previous studies having been carried out with the alkylated nitrogen derivatives.[20,21] Compound **12** was assigned on the basis of its ^{31}P NMR multiplet at 28 ppm,

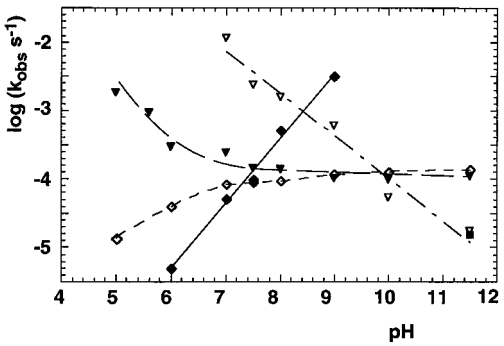


Figure 2. log k_{obs} (s^{-1}) versus pH for the hydrolysis of UpsU (◆), amUpsU (◇), UpnU (▼), amUpnT (▽) and UpU (■).

which is similar to the chemical shift of 20 ppm observed by Johnson *et al.*[22] for an N-methylated oxazaphospholidin-2-one ester. Compound **13** was assigned on the

Table 2. Dependence of the Rate of UpsU, UpnU, and amUpsU Hydrolysis with Metal Ions (10 mM, pH 7.5)[a]

	none	Mg^{2+}	Zn^{2+}	Cd^{2+}
UpsU	97	227	1300	803
UpnU	140	197	1491	1682
amUpsU	89	93	223	3083

[a] k_{obs} (10^{-6} s^{-1}); 15% error between experiments.

Table 3. Michaelis−Menten Parameters for UpU, UpsU, and UpnU as Substrates for Pancreatic Ribonuclease A

	k_{cat} (s^{-1})[c]	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})
UpU[a]	5.3	8	0.66
UpnU[a]	195	7.3	26.7
UpsU[b]	6.5	4	1.6

[a] HEPES (50 mM), NaCl (200 mM). [b] HEPES (500 mM). [c] k_{cat} 15% error between experiments.

basis of its ^{31}P NMR chemical shift of 7.5 ppm, similar to the chemical shifts of UpnU and TpnT at 9.3 and 8.4 ppm, respectively. This is consistent with our observation that there is not a big change in chemical shift between the phosphate monoester, uridine-2′(3′)-monophosphate (4 ppm), and the phosphate diester, UpU (0 ppm), at pH 10. The intermediacy of the cyclic phosphoramidate **12** is reasonable, particularly considering that both TpsT and TpnT, which have no 2′-hydroxyl group, are completely stable toward hydrolysis in the pH range from 5 to 11.5.

Dependence of Hydrolytic Stability upon the Presence of Divalent Metal Cations. The rates of hydrolysis of UpsU, amUpsU, and UpnU were also determined in the presence of Mg^{2+}, Zn^{2+}, and Cd^{2+} (Table 2), but no reliable data could be obtained for amUpnT due to its instability at pH 7.5. The hydrolysis rates in the absence of metal ions did not change upon addition of 10 mM EDTA, which implied that the buffers did not contain any metal ion contamination. In the presence of Mg^{2+}, there is a relatively small increase in the rate of hydrolysis with all three dinucleotides. In the presence of Zn^{2+} or Cd^{2+}, an increase in hydrolysis rate of ca. 10-fold is observed with UpsU and UpnU. In contrast, amUpsU hydrolysis was not significantly enhanced in the presence of either Mg^{2+} or Zn^{2+}; however, a 35-fold increase was observed in the presence of Cd^{2+}.

Substrate Properties with RNase A. The Michaelis−Menten parameters for the reaction of UpU, UpsU, and UpnU with RNase A were determined (Table 3). For UpU degradation k_{cat}/K_M is 0.66 mM^{-1} s^{-1} and is approximately 4-fold lower than that previously published, k_{cat} = 11 s^{-1}, K_M = 3.7 mM, and k_{cat}/K_M = 2.97 mM^{-1} s^{-1}.[23] Due to the presence of AcOH in our sample of UpsU, its kinetic determination was carried out in HEPES (pH 7.5, 500 mM), compared with HEPES (pH 7.5, 50 mM) and NaCl (200 mM) for UpnU and UpU. Hydrolysis of UpU using both buffer conditions gave values within experimental error. UpnU has a 40-fold higher catalytic efficiency as a result of the same increase in the value of k_{cat}. In contrast the 2-fold increase in catalytic efficiency observed for UpsU is mainly a result of its 2-fold lower K_M value. No data for amUpnT could be determined due to its instability at pH 7.5, where its rate of hydrolysis is approximately 1 order of magnitude faster than those of UpsU, amUpsU, and UpnU.

Discussion

Polyribonucleotides and oligoribonucleotides containing a 2′-amino group as opposed to a 2′-hydroxy group are stable to alkaline, RNaseA, and hammerhead ribozyme-induced hydrolyses.[9−12] This amino group should however be sufficiently nucleophilic to attack the phosphorus of the internucleotidic linkage in a transesterification reaction to yield the cyclic phosphoramidate. The pK_a of the 2′-amino group is 6.2,[17,18] and therefore protonation cannot be the cause for the observed stability above pH 7. We considered the properties of the leaving group to be important for a successful displacement reaction involving the 2′-amino group, and therefore the uridine dinucleotide analogues UpsU, amUpsU, UpnU, and amUpnT were prepared to systematically investigate their hydrolytic properties.

The hydrolysis of uridine dinucleotide UpU has been extensively studied in the past.[24−27] It is extremely stable toward acid−base hydrolysis with a $k_{obs} = 9.8 \times 10^{-9}$ s^{-1} at pH 8.36 and 41 °C.[28] As a result of this stability, elevated temperatures are required to obtain reliable kinetic data for hydrolysis at near neutral pH values.[26] In order to assess the influence of the leaving group on the hydrolysis/transesterification of UpU, we have changed the leaving group from a 5′-hydroxyl to a thiol and a primary amine. The hydrolysis rates of UpU and of these analogues are compared (Figure 2). The log k_{obs} vs pH profile for UpsU, in the range 6−9, indicates that UpsU hydrolysis is base-catalyzed, and this is indicative of the deprotonation of the 2′-hydroxyl group being rate-limiting. This is consistent to that observed for UpU at 90 °C[26] and uridine 3′-(*o*-carboxyphenyl) ester,[29] which were also base-catalyzed in this region and only became pH independent as the pH values neared the pK_a of the 2′-hydroxyl group, pK_a = 13.[30] Additionally, the hydrolysis of UpU, at pH 8,[28] is at least 5 orders of magnitude slower than that of UpsU, which is consistent with the differences between the rate of hydrolysis of alkyl S-phosphorothioate and phosphate esters.[31,32] Hydrolysis of amUpsU, in contrast to the stability of amUpT, was only pH dependent in the region from pH 5 to 7, above which the hydrolysis rate plateaus to its maximum value at pH 11.5, $k_{obs} = 138 \times 10^{-6}$ s^{-1}. The profile observed for amUpsU (Figure 2) correlates well with the pK_a of the 2′-ammonium group of 6.2 determined for amUpT.[17,18] Thus, as the pH decreases, the equilibrium shifts toward the protonated amine, which is not nucleophilic. However, above ca. 7.2, one unit above the pK_a, the amino species predominates and hydrolysis plateaus to its maximum rate.

(21) Brown, C.; Boudreau, J. A.; Hewitson, B.; Hudson, R. F. *J. Chem. Soc. Perkin Trans. 1* **1976**, 888−895.
(22) Johnson, C. R.; Elliott, R. C.; Penning, T. D. *J. Am. Chem. Soc.* **1984**, *106*, 5019−5020.
(23) Richards, F. M.; Wyckoff, H. W. In *The Enzyme*; Boyer, P. D., Ed.; Academic Press: New York 1971; Vol. IV, p 647−806.
(24) Butzow, J. J.; Eichhorn, G. L. *Biochemistry* **1971**, *10*, 2019−2027.
(25) Ikenaga, H.; Inoue, Y. *Biochemistry* **1974**, *13*, 577−582.
(26) Järvinen, P.; Oivanen, M.; Lönnberg, H. *J. Org. Chem.* **1991**, *56*, 5396−5401.
(27) Kuusela, S.; Lönnberg, H. *J. Chem. Soc. Perkin Trans. 1* **1994**, 2301−2306.
(28) Chapman, W. H. J.; Breslow, R. *J. Am. Chem. Soc.* **1995**, *117*, 5462−5469.
(29) Chandler, A. J.; Kirby, A. J. *J. Chem. Soc., Chem. Commun.* **1992**, 1769−1770.
(30) Narlikar, G. J.; Gopalakrishnan, V.; McConnell, T. S.; Usman, N.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3668−3672.
(31) Hudson, R. F.; Keay, L. *J. Chem. Soc. Perkin Trans. 1* **1956**, 3269−3271.
(32) Thain, E. M. *J. Chem. Soc. Perkin Trans. 1* **1957**, 4694−4699.

In light of the fact that nucleophilic displacement reactions with primary amines on phosphate diesters have been reported for the methyl 2,4-dinitrophenyl diesters at pH 10, a contributing factor in the hydrolysis of both UpsU and amUpsU is the pK_a of the leaving group. Such phosphoryl transfer reactions typically occur with rates of 8×10^{-2} M^{-1} min^{-1}, where the leaving group is 2,4-dinitrophenol, which has an extremely low pK_a of 4.[33] The dependence of rates on the pK_a of the leaving group has been demonstrated for reactions of nicotinamide with phosphate monoester monoanions, for which the nucleophilic substitution reaction is the same as for diester monoanions.[34] This dependence is also reported for the reaction of quinuclidine and pyridine nucleophiles with phosphate ester monoanions.[35] Thus, the hydrolysis of UpsU and amUpsU, compared to UpU and amUpT, is clearly more facile due to the differences in pK_a between the 5′-thio (pK_a = 11) and the 5′-oxo (pK_a = 16) leaving group.[36] This difference in hydrolytic activity can also be attributed to the much lower P−S bond strength, ca. 50 kcal mol^{-1}, compared to the P−O bond strength of ca. 91 kcal mol^{-1} and the P−N bond strength of ca. 70 kcal mol^{-1}.[37]

Hydrolysis of UpnU below pH 6 is acid-catalyzed but the rate of hydrolysis between pH 6 and 11.5 is constant, ca. 140×10^{-6} s^{-1} (Figure 2). A log linear increase in the hydrolysis of UpU[26] and uridine 3′-(*o*-carboxyphenyl) ester[29] was also observed in the acidic pH range down to pH 1, which was attributed to protonation of the phosphate diester with a pK_a of ca. 1.5.[38] With UpnU there exists the additional possibility for rate acceleration through protonation of the 5′-imino group, for which the pK_a is not known. The leveling off of the hydrolysis rate of UpnU above pH 6 is not clearly understood. One would have expected a log linear increase in the rate of hydrolysis as the pK_a of the 2′-hydroxyl group is approached, since this increases the concentration of the 2′-oxo anion species, which is more nucleophilic than a 2′-hydroxyl. However, at higher pH one would also expect a decrease in the rate as the protonation of the 5′-imino becomes more difficult, thus possibly canceling out any increase derived from the greater concentration of the 2′-oxo anion. The profile observed for amUpnT hydrolyis gives a straight line of gradient −0.7 (Figure 2), which supports the idea that protonation of the 5′-imino group is rate-limiting. It is more readily hydrolyzed than UpnU and it is not until above pH 10 that amUpnT exhibits greater hydrolytic stability than UpnU. Thus, the 2′-oxo anion in UpnU more readily displaces the 5′-amine than does the 2′-amino group in amUpnT, as expected from the difference in P-O and P−N bond strengths discussed above.

Clearly 5′-thiouridine is a better leaving group than uridine and two factors, the P−S bond strength and the lower pK_a of 5′-thiouridine of approximately 11 vs uridine of approximately 16, enable it to be displaced by a primary amine as a nucleophile in an intramolecular reaction. 5′-Aminouridine is also a much better leaving group than uridine, and hydrolysis is facilitate by the ready protonation of the 5′-imino function. Introduction of 2′-amino groups clearly stabilizes phosphodiester bond hydrolysis with both 5′-thio- and 5′-amino leaving groups, but only above pH's 7 and 10, respectively. This study demonstrates that the stability of 2′-amino containing poly- and oligoribonucleotides is due to the poor leaving characteristics of the 5′-nucleoside and the greater nucleophilicity of a 2′-oxo compared to a 2′-amine.

Metal Ion Dependence. The rate of hydrolysis of UpU[24,25,27,39] and phosphate diesters[29,39−42] is dramatically increased in the presence of divalent metal ions and the metal ion is thought to co-ordinate to the phosphate group. Zn^{2+} and Cd^{2+} were used in this study since their metal−aquo complexes have similar pK_a values, but Cd^{2+} is considered "softer" and therefore it should enable differentiation between activation of hydrolysis through general base catalysis and co-ordination to the 5′-thio leaving group. The observed rate enhancements in UpsU hydrolysis do not correlate with what would be expected from increased general-base catalysis, since the pK_a values are 11.4, 8.8, and 9.0 for the aquo/hydroxy complexes of Mg^{2+}, Zn^{2+}, and Cd^{2+}, respectively.[43] Additionally, on the basis of the "hardness" and "softness" of Zn^{2+} and Cd^{2+}, respectively, one would have expected an enhancement in UpsU hydrolysis in the presence of Cd^{2+}. The ca. 2-, 11-, and 12-fold rate enhancements observed for UpsU and UpnU hydrolysis in the presence of Mg^{2+}, Zn^{2+}, and Cd^{2+}, respectively, is comparable to that observed for UpU hydrolysis[27] and does not correlate with increased general-base catalysis.

Metal ion dependent hydrolysis of amUpsU is potentially the most interesting, since the general-base properties of the metal ion−aquo complex should not be important because the 2′-amino group is mainly deprotonated at pH 7.5 and does not require activation by a metal ion to enable nucleophilic attack on the phosphorus. Thus activation of the 5′-thio leaving group by a "soft" metal ion might be most easily visible. The increase in rate of hydrolysis from Mg^{2+} to Zn^{2+} is 2-fold and is another 15-fold to Cd^{2+}. This is the effect one would expect if there was rate acceleration by the soft metal ion co-ordinating to the soft thio leaving group. There are no numbers available for the preference of co-ordination of metal ions to oxygen vs sulfur except for the difference in interaction with ATP and ATPβS.[44] There it is concluded that the ratio of oxygen vs sulfur coordination is 31 000 for Mg^{2+}, 3100 for Ca^{2+} , 160 for Mn^{2+}, and 0.018 for Cd^{2+}.

It is very difficult to establish exactly how metal ions promote hydrolysis of dinucleotides, as several effects may contribute to the rate enhancement. (1) The metal ion aquo−hydroxy complex can enhance deprotonation or protonation by general-acid−base catalysis. (2) The metal ion could co-ordinate to the phosphate acting as a Lewis acid, lowering the negative charge on the phosphate group, making it more susceptible to nucleophilic

(33) Kirby, A. J.; Younas, M. *J. Chem. Soc. (B)* **1970**, 510−513.
(34) Kirby, A. J.; Varvoglis, A. G. *J. Chem. Soc. (B)* **1968**, 135−141.
(35) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 7587−7596.
(36) March, J. *Advanced Organic Chemistry*, 4th ed.; John Wiley and Sons: New York, 1992.
(37) Hudson, R. F. *Structure and Mechanisms in Organo-Phosphorus Chemistry*; Academic Press: London, 1965.
(38) Kumler, W. D.; Eiler, J. J. *J. Am. Chem. Soc.* **1943**, *65*, 2355−2361.
(39) Breslow, R.; Huang, D.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4080−4083.
(40) Breslow, R.; Berger, D.; Huang, D.-L. *J. Am. Chem. Soc.* **1990**, *112*, 3686−3687.
(41) Browne, K. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1992**, *114*, 4951−4958.
(42) Dempcy, R. O.; Bruice, T. C. *J. Am. Chem. Soc.* **1994**, *116*, 4511−4512.
(43) Basolo, F.; Pearson, R. *Mechanismen in der Anorganischen Chemie*; Georg Thieme Verlag: Stuttgart, 1973.
(44) Pecoraro, V. L.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5262−5271.

attack from the 2′-hydroxyl. This is similar to protonation of the phosphate diester, which has been previously attributed to the increase in hydrolysis of UpU[26] and uridine 3′-(*o*-carboxyphenyl) ester[29] under acidic conditions. (3) Direct co-ordination of the metal ion to the leaving group would stabilize the bond cleavage step without requiring protonation, as proposed for the hammerhead[45] and *tetrahymena*[46] ribozymes. Care must also be taken when extrapolating data obtained with activated esters, since clearly they can hydrolyze by a different mechanism as a result of the lower pK_a of the leaving group. In a study on the cleavage of a hammerhead ribozyme substrate containing a 5′-thio leaving group, it was concluded that no metal ion was bound to the leaving group in the transition state. This does not however imply that the native hammerhead ribozyme, which contains a 5′-oxo leaving group, does not bind a metal ion to stabilize the leaving group, since the rate-determining step for the hydrolysis reaction may be different for the two substrates on account of the different pK_a's of the leaving group. Thus, the 5′-thio leaving group may not need to be stabilized by protonation or metal ion co-ordination. This difficulty in attributing rate enhancements to a particular effect is exemplified by the hydrolysis of various XpXp(2′) dinucleotides with Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}, the four metal ions for which data is available.[24] The rates are all within 1 order of magnitude of each other and therefore it seems that the structure of the metal ion/nucleotide complex is more important for the rate of reaction than enhanced deprotonation of the 2′-hydroxyl group. Thus, the metals enhance hydrolysis irrespective of the leaving group for UpsU and UpnU, indicating a similar mode of enhancement for both.

The results presented here for amUpsU hydrolysis are consistent with the concept that Cd^{2+} can stabilize the developing negative charge on the 5′-thio- leaving group; however, it is not clear why this enhancement does not also manifest itself in UpsU hydrolysis. Thus, the data presented here should caution the interpretion of results in terms of particular interactions as at present a clear understanding of metal ion-catalyzed hydrolysis of RNA or oligoribonucleotides is far from complete.

RNase-Catalyzed Hydrolysis. The above compounds were also tested as substrates for pancreatic ribonuclease (RNase A) which catalyses RNA phosphodiester bond cleavage 3′ to pyrimidine nucleosides forming 2′,3′-cyclic phosphate and 5′-hydroxy termini.[23] Of the five dinucleotides investigated only UpU, UpnU, and UpsU were cleaved, but amUpsU and amUpnT were not. The three cleavable dinucleotides had only marginal differences in their K_M values (Table 3), which suggests that association of the substrate with the enzyme in the ground state is approximately the same for all of them. The k_{cat} values for UpU and UpsU were also similar. However, UpnU displays a k_{cat} value 35-fold higher than that observed for UpsU and UpU. This argues that UpnU degradation by RNase A is enhanced, compared to UpU and UpsU, because it is more easily protonated in the active site of the enzyme by the general acid His-119. In contrast to UpU, which is only acid-catalyzed below pH 5[26] the hydrolysis of UpnU is acid-catalyzed below ca. pH 7 and incorporation of a phosphoramidate linkage into the cleavage site of ribozymes would be a suitable modification to probe the sensitivity of the leaving group toward protonation. If protonation does operate in the cleavage mechanism of ribozymes, one would expect to see an increase in the rate of cleavage at pH 7 and a change in the pH vs rate profile.

It is interesting to note that amUpsU is not a substrate for RNaseA whereas UpsU is, indicating that P−S bond cleavage is not a problem. Although it is not possible at present to identify the reason for this stability, one might speculate that the 2′-amino group is not placed in the correct orientation for the reaction to proceed, as the 2′-amino nucleoside is predominantly in the 2′-endo conformation and not the 3′-endo conformation usually adopted by the ribonucleosides.

Conclusions

We have undertaken the synthesis of the uridine dinucleotides UpsU, amUpsU, UpnU, and amUpnT and studied their hydrolytic properties under various conditions. These dinucleotides are readily hydrolyzed in the pH range from 5 to 11.5. with the most important finding being that amUpsU and amUpnT are hydrolyzed in contrast to amUpT, indicating the importance of the leaving group when the 2′-amino group is the nucleophile. The metal ion effect of Cd^{2+} on the hydrolysis of amUpsU is a clear example for metal ion interaction with the leaving group. UpnU demonstrated a marked rate enhancement, relative to UpU and UpsU, when cleaved by RNase A, consistent with the postulated protonation of the 5′-amino leaving group in the hydrolysis of UpnU and amUpnT.

Experimental Section

Materials and Methods. Ribonuclease inhibitor (110 units/mL) was purchased from United States Biochemicals and bovine pancreatic ribonuclease (RNase A) (DNase free) was obtained from Boehringer Mannheim. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*O*-(*β*-cyanoethyl *N*,*N*′-diisopropylphosphoramidite) and 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-*O*-(*β*-cyanoethyl *N*,*N*′-diisopropylphosphoramidite) were obtained from Applied Biosystems (Dreieich, Germany) and Milliore BioSyntech (Hamburg, Germany), respectively. 5′-Iodo-5′-deoxyuridine,[47] 5′-azido-5′-deoxyuridine,[48] 5′-amino-5′-deoxyuridine,[49] 5′-thio-5′-deoxyuridine,[50] 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxy-2′-[(trifluoroacetyl)amino]-uridine 3′-*O*-(*β*-cyanoethyl *N*,*N*′-diisopropylphosphoramidite),[9] thymidylyl (3′−5′) 5′-amino-5′-deoxythymidine (TpnT),[51] and 2′-amino-2′-deoxyuridylyl (5′−3′)-thymidine[17] were prepared as previously published. All other reagents were purchased commercially and used as received except for dry pyridine, 1,4-dioxane, and tetrahydrofuran, which were stored over 4 Å molecular sieves. CH_2Cl_2 used in phosphitylation reactions was stored over sodium−lead alloy (10% Na, Merck) and passed down a column of basic alumina prior to use. Analytical thin layer chromatography (TLC) was carried out using silicagel 60 F_{254} plates (Merck), which were developed with one of the following solvent systems: solvent A, CH_2Cl_2/EtOAc/Et_3N (47.5:47.5:5 v/v); solvent B, CH_2Cl_2/EtOAc/Et_3N (5:90:5 v/v); solvent C, CH_2Cl_2/MeOH (90:10 v/v); solvent D, CH_2Cl_2/

(45) Sawata, S.; Komiyama, M.; Taira, K. *J. Am. Chem. Soc.* **1995**, *117*, 2357−2358.
(46) Piccirilli, J. A.; Vyle, J. S.; Caruthers, M. H.; Cech, T. R. *Nature* **1993**, *361*, 85−88.
(47) Tsuji, T.; Takenaka, K. *Nucleosides Nucleotides* **1987**, *6*, 575−580.
(48) Yamamoto, I.; Sekine, M.; Hata, T. *J. Chem. Soc. Perkin Trans. 1* **1980**, 306−310.
(49) Saneyoshi, M.; Fujii, T.; Kawaguchi, T.; Sawai, K.; Kimura, S. In *Nucleoside Chemistry (part 4)*; Townsend, L. B. a. T., L. S., Ed.; J. Wiley: New York, 1987.
(50) Reist, E. J.; Benitez, A.; Goodman, L. *J. Org. Chem.* **1964**, *29*, 554−558.
(51) Mag, M.; Engels, J. W. *Tetrahedron* **1994**, *50*, 10225−10234.

MeOH (80:20 v/v); solvent E, i-PrOH/NH_3/H_2O (55:35:10 v/v). Column chromatography was performed on silica gel 60 (Merck) of particle size 0.063−0.200 mm.

Mass spectral[52] and NMR[53] analyses were carried out as previously described. Analytical and preperative HPLC were carried out using the following buffer systems. Buffer system 1: buffer A, TEAA (50 mM); buffer B, TEAA (50 mM)/CH_3CN (70% v/v). Buffer system 2: buffer A, TEAB (100 mM); buffer B, TEAB (100 mM)/CH_3CN (70% v/v). Gradient 1, 20 min linear gradient from 100% to 80% buffer A; gradient 2, 40 min linear gradient from 100% to 50% buffer A; gradient 3, 25 min linear gradient from 80% to 0% buffer A.

Kinetic Analysis for the Hydrolytic Degradation of Dinucleotides. The rate of dinucleotide hydrolysis was established by fitting the fraction of remaining substrate, relative to an internal standard, to a single exponential decay. Buffers were as follows, with pH adjusted at 37 °C: sodium acetate (pH 5, 100 mM); Na_2HPO_4−citric acid (pH 6, 100 mM); HEPES (pH 7 to 8, 100 mM); MES (pH 9, 100 mM); glycine−NaOH (pH 9, 100 mM); carbonate−bicarbonate (pH 10, 100 mM) and disodium phosphate (pH 11.5, 100 mM), NaCl solution was used to adjusted ionic strength to 1 M. Reaction mixtures were prewarmed at 37 °C before the reaction was initiated by addition of the dimer solution containing the internal standard nucleoside. Concentrations of dimer and internal standard were UpnU (400 μM) and ddT (800 μM), UpsU (200 μM) and ddA (800 μM), amUpsU (400 μM) and dA (800 μM), or amUpnT (200 μM) and dA (400 μM). With the exception of amUpnT hydrolysis, aliquots (5 μL) were removed at appropriate time points, diluted with TEAA buffers (50 mM, 40 μL), frozen at liquid nitrogen temperature, and maintained at −80 °C until required for analysis and the fraction of remaining dinucleotide established by HPLC analysis (buffer system 1, gradient 1). For amUpnT the aliquots were diluted with glycine−NaOH buffer (pH 12, 500 mM, 40 μL) and analysed as above. Dependence of the hydrolysis rates upon the buffer concentration was assessed in the above manner as described above using HEPES (pH 7.5), which was varied from 50 to 500 mM. Dependence of hydrolysis rates upon the presence of metal ions was also carried out as described above using HEPES (pH 7.5, 100 mM) in the presence of the relevant metal ion (10 mM) and reaction was quenched with EDTA (50 mM, pH 7.5., 10 μL), frozen in liquid nitrogen, and maintained at −80 °C until required for HPLC. Duplication of the hydrolysis reactions gave an error of 15% for the values of k_{obs}.

Michaelis−Menten Parameters for RNase A Cleavage of the Dinucleotides. Kinetic constants k_{cat} and K_M were determined from Eadie−Hofstee plots obtained from initial velocities, at various substrate concentrations, under multiple turnover conditions. Nucleotide dimer concentrations of 0.5−6 mM were used and the RNase A concentration was varied from 18.25 to 365 nM, depending upon the rate of the reaction. The concentration of internal standard, ddT for hydrolysis with UpnU and UpU, ddA for hydrolysis with UpsU, and dA for hydrolysis with amUpsU, was from 2 to 8 mM. For UpU and UpnU a solution containing the nucleotide dimer, internal standard, HEPES (pH 7.5, 50 mM), and NaCl (200 mM) was incubated for 10 min at 25 °C and the reaction initiated by addition of RNase A. For UpsU a solution containing the nucleotide dimer, internal standard, and HEPES (pH 7.5, 500 mM) was incubated for 10 min at 25 °C and the reaction initiated by addition of RNase A. Aliquots (5 μL) were removed at appropriate time points, quenched by addition to a solution of RNAse inhibitor (1.6 units/mL), HEPES (pH 7.5, 20 mM), KCl (50 mM), dithiothreitol (5 mM), and glycerol (50%), and maintained at −80 °C. Initial rates were established by quantifying the amount of starting material remaining, with respect to the internal standard, by HPLC analysis (buffer system 1, gradient 1) of the collected time points. Duplication of the RNase A-induced degradation reactions gave an error of 15% for the values of k_{obs}.

2′-*O*-(*tert*-Butyldimethylsilyl)uridine 3′-phosphorothioate (1). 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*O*-(β-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (500 mg, 580 μmol) was dissolved in anhydrous CH_3CN (3 mL) under an argon atmosphere. A solution of tetrazole, in CH_3CN (0.5 M, 1.5 mL, 1.3 equiv), and 3-hydroxypropionitrile (44 μL, 1.2 equiv) was then added, and the mixture stirred at room temperature for 1.5 h, R_f (starting material) 0.8 (solvent A). The reaction mixture was then added, under argon, to 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[54] dissolved in CH_3CN, (0.2 M, 15 mL), and stirring continued for a further 2.5 h. The solution was concentrated *in vacuo* and the residue was suspended in H_2O and extracted with ether (3 × 60 mL), and the organic phase was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was then suspended in MeOH (15 mL)/ammonia (32%, 56 mL) and the mixture heated at 55 °C for 2 h. After cooling on ice for 15 min, this mixture was concentrated *in vacuo*, AcOH (80%) was added, and the mixture was kept at room temperature for 30 min before being evaporated to dryness. The residue was then dissolved in H_2O (40 mL) and washed with ether (2 × 40 mL), and the aqueous phase was evaporated to dryness. The white crude product was then dissolved in a small volume of H_2O and purified by chromatography using DEAE-Sephadex A-25 eluted with a linear gradient of TEAB (0 to 0.5M, total volume 3 L). This yielded the disulfide-bridged nucleotide dimer, which after concentration *in vacuo* was taken up in H_2O (2.8 mL), treated with an aqueous solution of dithiothreitol (1 M, 100 μL), and maintained at room temperature for 4.5 h. The mixture was subsequently evaporated to dryness, dissolved in TEAB (100 mM, 1 mL), and purified by chromatography over DEAE-Sephadex A-25 eluted with a linear gradient of TEAB (0−0.5 M, total volume 1 l) to yield **1** (1170 A_{260} units, 117 μmol, 20%): ^{31}P NMR (D_2O) 56 ppm; MS *m*/*z* 453.093 483, calcd for $C_{15}H_{26}N_2O_8S_1P_1Si_1$ 453.091 680.

2′-*O*-(*tert*-Butyldimethylsilyl)uridylyl (3′−5′) 5′-Thio-5′-deoxyuridine. Compound **1** (1170 A_{260} units, 117 μmol) was loaded onto a Merck I ion exchange resin (pyridinium form) column (2 × 16 cm) and eluted with H_2O (400 mL) into a solution of tri-*n*-butylamine (254 μmol, 3 equiv, 90 μL) in MeOH (3 mL). This solution was then evaporated to dryness and the residue redissolved in MeOH (5 mL). The tri-n-butylammonium salt of 2′-(*O*-*tert*-butyldimethylsilyl)uridine 3′-monophosphate (500 A_{260} units, 50 μmol, 2.12 mL) was then evaporated to dryness, the residue dried by twice evaporation from anhydrous *N*,*N*′-dimethylformamide (2 mL) and then dissolved in anhydrous *N*,*N*′-dimethylformamide (1 mL). 5′-Iodo-5′-deoxyuridine (35.3 mg, 2 equiv) and tri-n-butylamine (36 μL, 3 equiv) were then added. The reaction was then maintained at room temperature for 48 h, after which conversion to product was quantitative, as observed by ^{31}P NMR. The reaction mixture was evaporated to dryness, dissolved in H_2O (5 mL), and purified by chromatography over DEAE-Sephadex A-25 eluted with a linear gradient of TEAB (0−0.5 M, total volume 1 l) to yield the product (598 A_{260} units, 27 μmol, 50%): HPLC t_R (buffer system 1, gradient 2) 27.5 min; ^{31}P NMR (D_2O) 20.4 ppm; MS *m*/*z* 679.148 956, calcd for $C_{24}H_{36}N_4O_{13}S_1P_1Si_1$ 679.150 651.

Uridylyl (3′−5′) 5′-Thio-5′-deoxyuridine (UpsU). 2′-*O*-(*tert*-Butyldimethylsilyl)uridylyl (3′−5′) 5′-thio-5′-deoxyuridine (300 A_{260} units, 15 μmol) in H_2O (1.3 mL) was evaporated to dryness, dried by twice evaporation from tetrahydrofuran (3 mL), dissolved in triethylamine trihydrogen fluoride (1 mL), and maintained at room temperature for 22 h. The product was then purified by preparative HPLC (buffer system 2, gradient 2) followed by lyophilization to yield a white solid (135 A_{260} units, 6.8 μmol, 45%): HPLC t_R (buffer system 1, gradient 2) 9.6 min; ^{31}P NMR (D_2O) 18.8 ppm; MS *m*/*z* 565.064 941, calcd for $C_{18}H_{22}N_4O_{13}S_1P_1$ 565.064 172.

2′-Amino-2′-deoxyuridine 3′-Phosphorothioate (2). This was prepared by the same method described for compound **1** but using 5′-*O*-(4,4′-dimethoxytrityl)-2′-(trifluoroacetamido)-2′-deoxyuridine 3′-*O*-(β-cyanoethyl *N*,*N*-diisopropylphosphor-

(52) Eckart, K.; Spiess, J. *Am. Soc. Mass Spectrom.* **1995**, *6*, 912−919.

(53) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1991**, *56*, 1777−1783.

(54) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693−4699.

amidite) (420 mg, 500 μmol) and S_8 dissolved in CS_2/pyridine (1:1) for oxidation to give **2** (2540 A_{260} units, 254 μmol, 51%): ^{31}P NMR (D_2O) 44.45 ppm; MS *m/z* 338.020 500, calcd for $C_9H_{13}N_3O_7P_1S_1$ 338.021 185.

2′-Amino-2′-deoxyuridylyl (3′−5′) 5′-Thio-5′-deoxyuridine (amUpsU). This was prepared by the same method described for 2′-*O*-(*tert*-butyldimethylsilyl)uridylyl (3′−5′) 5′-thio-5′-deoxyuridine but using compound **2** (500 A_{260} units, 50 μmol), tri-n-butylamine (36 μL, 3 equiv), and 5′-iodo-5′-deoxyuridine (35 mg, 2 equiv, 100 μmol) to give amUpsU (496 A_{260} units, 24.8 μmol, 49.6%): ^{31}P NMR (D_2O) 20.16 ppm; MS *m/z* 564.079 086, calcd for $C_{18}H_{23}N_5O_{12}S_1P_1$ 564.080 157.

Thymidine (3′−5′) 5′-Thio-5′-deoxythymidine (TpsT). This was prepared by the same method described for amUpsU but using thymidine 3′-thiomonophosphate (125 A_{267} units, 12.5 μmol), tri-n-butylamine (9 μl, 3 equiv), and 5′-iodo-5′-deoxythymidine (9 mg, 2 equiv 25 μmol) to give TpsT (125 A_{267} units, 6.8 μmol, 50%): ^{31}P NMR (D_2O) 19.80 ppm; MS *m/z* 561 ($M - H^+$).

(R_P/S_P)-5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridylyl (3′−5′)-5′-Amino-5′-deoxyuridine Methyl Ester (3). 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine (1.336 g, 2 mmol) was dissolved in dry CH_2Cl_2/CH_3CN (10:7, v/v, 17 mL), and the flask was sealed with a septum and flushed with argon. To this was then added a solution of tetrazole dissolved in CH_3CN (0.5 M, 3 mL, 0.75 equiv) and (*N*,*N*′-diisopropylamino)dimethoxyphosphane (0.404 g, 2.09 mmol, 1.05 equiv), and the solution was stirred for 4 h at room temperature. With maintenance of the argon atmosphere, anhydrous ether (50 mL) was then added and the resultant white precipitate removed by filtration. The supernatant was concentrated *in vacuo* and the residue dissolved in anhydrous pyridine (5 mL). To this solution was added LiCl (0.420 g, 10 mmol, 5 equiv) followed by 5′-azido-5′-deoxyuridine (0.875 g, 3.25 mmol, 1.62 equiv), and the reaction mixture was stirred for 20 h at room temperature, R_f (product) 0.25 (solvent C). The crude product was then concentrated, *in vacuo*, and purified by silica gel column chromatography, eluting with CH_2Cl_2 (0%−10% MeOH) containing triethylamine (1%), followed by preparative HPLC [(buffer system 2, gradient 3) t_R (**3**) 16 min, t_R (5′-azido-5′-deoxyuridine) 6 min] to yield **3** (5560 A_{260} units, 278 μmol, 14%). ^{31}P NMR (CD_3OD) 12.30 (s) and 12.66 (s), 1:1; *m/z* 978.336 304, calcd for $C_{46}H_{57}N_5O_{15}P_1Si_1$ 978.335 809.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridyl (3′−5′) 5′-Amino-5′-deoxyuridine (4). Compound **3** (1000 A_{260} units, 50 μmol), in MeOH (1.8 mL), was evaporated to dryness and evaporated twice from dioxane (0.5 mL). To this residue was then added a solution of thiophenol/triethylamine/dioxane (1:2:3, v/v, 6 mL) and the resulting solution stirred at room temperature for 24 h; TLC (solvent D) R_f (**4**) 0.4, R_f (**3**) 0.68. The reaction mixture was concentrated *in vacuo*, the residue was twice evaporated from MeOH (2 mL), and the crude product was purified by silica gel chromatography, eluting with CH_2Cl_2 (0%−10% MeOH) containing triethylamine (1%), to yield **4** (871 A_{260} units, 43.5 μmol, 87%): ^{31}P NMR (CD_3OD) 8.4; MS *m/z* 964.320 159, calcd for $C_{45}H_{55}N_5O_{15}P_1Si_1$ 964.320 159.

2′-*O*-(*tert*-Butyldimethylsilyl)uridylyl (3′−5′) 5′-Amino-5′-deoxyuridine (5). To **4** (600 A_{260} units, 30 μmol) was added acetic acid (80%, 200 μL) and the solution was thoroughly shaken by vortexing. After 6 min, removal of DMTr was quantitative by HPLC [(buffer system 1, gradient 2) t_R (**5**) 17 min, t_R (**4**) 25 min] and the reaction mixture was cooled in an ice bath and brought to pH 7 by addition of NaOH (1.2 mL, 2 M). The reaction mixture was purified by chromatography over DEAE-Sephadex A-25 eluted with a linear gradient of TEAB (0 to 0.5M, total volume 1 l) to yield **5** (340 A_{260} units, 17 μmol, 57%): ^{31}P NMR (CD_3OD) 9.74; MS *m/z* 662.188 828, calcd for $C_{24}H_{37}N_5O_{113}P_1Si_1$ 662.189 479.

Uridylyl (3′−5′) 5′-Amino-5′-deoxyuridine UpnU. Compound **5**, dissolved in H_2O (0.9 mL) (300 A_{260} units, 15 μmol), was evaporated to dryness, the residue evaporated twice from THF (500 μL), TBAF (500 μL, 1.1 M) added, and the solution incubated for 16 h at rt. Subsequent analytical HPLC indicated that the reaction had gone to 90% completion [(buffer system 1, gradient 2,) t_R (UpnU) 8 min, t_R (**5**), 25 min]. Purification was carried out by preparative HPLC (buffer system 2, gradient 1), and the appropriate fractions were collected and frozen before being combined and lyophilized to yield UpnU as a white solid containing ca. 10% 2′,3′cUMP. The reaction product was dissolved in H_2O (2 mL) and stored at −20 °C (225 A_{260} units, 11.3 μmol, 75%): ^{31}P NMR (D_2O) 9.29 (s), [cUMP 20.83 (s)]; MS *m/z* 548.101 456, calcd for $C_{18}H_{23}N_5O_{13}P_1$ 548.103 000.

(R_P/S_P)-5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxy-2′-(trifluoroacetamido)uridylyl (3′−5′) 5′-Amino-5′-deoxythymidine Methyl Ester (6). This was prepared by the same method described for compound **3** but using 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxy-2′-(trifluoroacetamido)uridine (1.364 g, 2.12 mmol) dissolved in CH_2Cl_2:CH_3CN (1:1, v/v, 40 mL), tetrazole (0.5 M, 6.36 mL, 1.5 eq), (*N*,*N*′-diisopropylamino)-dimethoxyphosphane (0.613 g, 3.18 mmol, 1.5 eq), LiCl (0.445 g, 10.6 mmol, 5 eq), and 5′-azido-5′deoxythymidine (0.566 g, 2.12 mmol, 1 eq). The reaction mixture was concentrated *in vacuo* twice from toluene (2 × 10 mL), and the residue was dissolved in EtOAc (300 mL) and subsequently washed with 5% $NaHCO_3$ (100 mL) and NaCl (saturated 100 mL), dried (Na_2SO_4), filtered, and evaporated to dryness. The crude product was purified by silica gel chromatography followed by preperative HPLC to give **6** (8060 A_{260} unit 403 μmol, 19%): ^{31}P NMR (CD_3OD) 12.58 (s) and 12.66 (s), 1:1; MS *m/z* 957.268 193, calcd for $C_{43}H_{46}N_6O_{14}P_1F_3$ 957.268 349.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-deoxy-2′-(trifluoroacetamido)uridylyl (3′−5′) 5′-Amino-5′-deoxythymidine (7). This was prepared by the same method as described for compound **4** but starting with compound **6** to give **7** (89%): ^{31}P NMR (CD_3OD) 8.5; MS *m/z* 943.255 030, calcd for $C_{42}H_{44}N_6O_{14}P_1F_3$ 943.2526 992.

2′-Deoxy-2′-(trifluoroacetamido)uridylyl (3′−5′) 5′-Amino-5′-deoxythymidine (8). This was prepared by the same method as described for compound **5** but starting with compound **7** to give **8** (93%): ^{31}P NMR (CD_3OD) 9.71; MS *m/z* 641.122 494, calcd for $C_{21}H_{26}N_6O_{12}P_1F_3$ 641.122 019

2′-Amino-2′-deoxyuridylyl (3′−5′) 5′-Amino-5′-deoxythymidine (amUpnT). To a solution of compound **8** in MeOH (600A_{260} units, 30 μmol, 1 mL) was added Na_2CO_3 (10%, H_2O/MeOH 8:2, v/v, 6 mL), and the mixture was stirred for 8 h at rt. Subsequent analytical HPLC indicated that the reaction had gone to 80% completion; (buffer system 1, gradient 1,) t_R (amUpnT) 10.55 min, t_R (**8**), 15.2 min. Purification was carried out by preparative HPLC (buffer system 2, gradient 1), and the appropriate fractions were collected and frozen before being combined and lyophilized to yield amUpnT as a white solid contaminated with ca. 12% of cyclic phosphoramidate (**12**). The product was dissolved in H_2O (2 mL) and stored at −20 °C (380 A_{260} units, 19 μmol, 63%): ^{31}P NMR (D_2O + H_2O, pH 12) 9.04 (s), [28.8, s, cUMnP; 7.69, s, 2′-amino-2′-deoxyuridine 2′-monophosphoramidate].

2′-Amino-2′-deoxyuridine 3′-Monophosphate (9). 5′-(4,4′-Dimethoxytrityl)-2′-deoxy-2′-(trifluoroacetamido)-2′-deoxyuridine 3′-*O*-(β-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (210 mg, 250 μmol) was dissolved in anhydrous CH_3CN (3 mL) under an argon atmosphere. A solution of tetrazole in acetonitrile (0.5 M, 0.75 mL, 1.25 equiv) and 3-hydroxypropionitrile (20.38 μL, 1.2 eq) was then added and the mixture stirred at room temperature for 1.5 h. A solution of iodine (0.5 M, ca. 0.8 mL) in THF/pyridine/H_2O (7:2:1) was added dropwise to the reaction mixture until the color persisted, and stirring was continued for a further 30 min. $NaHSO_3$ (5%, 1.2 mL) was then added and the solution concentrated *in vacuo*. The residue was then dissolved in H_2O (50 mL) and extracted with CH_2Cl_2 (2 × 50 mL), and the organic phase was dried (Na_2SO_4), filtered, and evaporated to dryness. Removal of the protecting groups was performed as described for the synthesis of **2** to give compound **9** (1035 A_{260} units, 103 μmol, 41%): ^{31}P NMR (D_2O) 4.16 (d) J_{PH} = 8.24 Hz; MS *m/z* 322.043 045 $(M - H)^-$ calcd for $C_9H_{14}N_3O_8.P_1$ 322.044 028.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic

acid; TBAF, tetrabutylammonium fluoride; PEG, polyethylene glycol; PPG polypropylene glycol; TBDMSCl, tetrabutyldimethylsilyl chloride; tetrahydrofuran, THF; TEAA, triethylammonium acetate; TEAB, triethyl ammonium bicarbonate; UpnU, uridylyl (3′−5′) 5′-amino-5′-deoxyuridine; UpsU, uridylyl (3′−5′) 5′-thio-5′-deoxyuridine; TpnT, thymidylyl (3′−5′) 5′-amino-5′-deoxythymidine; amUpsU, 2′-amino-2′-deoxyuridylyl (3′−5′) 5′-thio-5′-deoxyuridine; amUpT, 2′-amino-2′-deoxyuridylyl (3′−5′) thymidine; TpsT, thymidylyl (3′−5′) 5′-thio-5′-deoxythymidine; t_R, retention time; ddT, 2′,3′-dideoxythymidine; ddA, 2′,3′-dideoxyadenosine; dA, 2′-deoxyadenosine.

Acknowledgment. We are very grateful to S. Th. Sigurdsson and P. A. Heaton for the critical reading of the manuscript and many stimulating discussions. We also thank U. Kutzke for valuable and expert technical assistance and B. Seeger for NMR spectroscopy. Support by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie is acknowledged. V. J. thanks the Deutscher Akademischer Austauschdienst for a short term fellowship.

JO960795L