Base Catalysis and Leaving Group Dependence in Intramolecular Alcoholysis of Uridine 3′-(Aryl phosphorothioate)s

Helena Almer and Roger Strömberg*

Contribution from the Arrhenius Laboratory, Department of Organic Chemistry, Stockholm University, S-10691 Stockholm, Sweden

Received October 6, 1995[⊗]

Abstract: Base-catalyzed intramolecular transesterification in uridine 3′-(aryl phosphorothioate)s (Up(S)Ar) has been studied with respect to the dependence on the acidity of the conjugate acid of the leaving aryloxy group (pK_a 7.1−10) as well as on the basicity of the catalyst (pK_a(BH) = 7−10.2). The synthesis of the studied phosphorothioates was accomplished by using a method based on condensation of a protected uridine 3′-H-phosphonate with the appropriate phenols. The rate constants for hydroxide and imidazole catalysis (25 °C, 0.25 M ionic strength) obey Brønsted linear free energy relationships and the obtained $\beta_{\text{leaving group}}$ (β_{lg}) values are −0.55 and −0.63, respectively. General-base-catalyzed release of 4-nitrophenoxide from the corresponding phosphorothioate also obeys a Brønsted relationship with respect to the basicity of the catalyst ($\beta = 0.59$). Rates of reactions of the phosphorothioates are somewhat lower than for the corresponding phosphates ($k_{(\text{UpAr})}/k_{(S_P-\text{Up(S)Ar})} \approx 1.7-3.6$ and $k_{(\text{UpAr})}/k_{(R_P-\text{Up(S)Ar})} \approx 1.2-2.6$ (the spatial arrangement of phosphorus ligands in the S_P isomer of Up(S)Ar are the same as in the R_P isomer of a dinucleotide). Leffler α values of 0.59 for proton abstraction and 0.36 for bond breaking to the leaving group in the transition state do not balance (imbalance in $\alpha = 0.23$), indicating some negative charge buildup on the central group of atoms in the transition state. The intramolecular transesterification in uridine 3′-(aryl phosphorothioate)s is considered to be a concerted associative process with a mechanism that is similar to that in the corresponding phosphate esters, the difference being that the transition state appears to have a slightly more dissociative character for the phosphorothioates.

Introduction

The most commonly used phosphate-modified analogues of mono-, di-, and oligonucleotides are nucleoside phosphorothioates with sulfur in the position of a nonbridging oxygen. Their use as tools in a vast number of biological investigations, in particular determination of the stereochemistry of enzyme-catalyzed reactions, has been extensively reviewed.[1−5] Since phosphorothioate analogues of oligonucleotides have a generally higher stability toward enzymatic degradation, they are also promising candidates for use in oligonucleotide therapeutics.[6,7]

The use of phosphorothioate analogues in elucidation of enzyme action is not only confined to stereochemical analysis. Sulfur substitution perturbs the chemical reactivity of the phosphate linkage, and this property has been used to probe which step is rate-limiting in enzyme-catalyzed phosphate transfer. Examples of this usage can be found in studies of DNA polymerase[8] and the L-21 Sca1 ribozyme[9,10] derived from a *Tetrahymena thermophilus* group I intron. Kinetic data with phosphorothioate analogues have also been used to analyze and elucidate details of the mechanisms of reactions catalyzed by enzymes such as RNAse A[11] and alkaline phosphatase.[12,13]

The data available on chemical hydrolysis and intramolecular alcoholysis are however quite sparse. Until recently one was mainly compelled to use values from hydrolysis of simple phosphorothioate triesters,[14,15] the monoester 4-nitrophenyl phosphorothioate,[12] uridine 2′,3′-cyclic phosphorothioate,[16] and a dinucleotide in 0.5 M sodium[17] or potassium hydroxide.[18] Studies on the rate of intramolecular alcoholysis in both the R_P and S_P diastereoisomers of uridine 3′-(uridine 5′-phosphorothioate) over a pH range of 9−12 were later reported.[19] These revealed that the effect of a nonbridging sulfur on the reaction rate was relatively small ($k_{\text{phosphate}}/k_{R_P-\text{thioate}} = 1.3$ and $k_{\text{phosphate}}/k_{S_P-\text{thioate}} = 0.78$).[19] This was constant over the investigated pH range, and the reaction is first-order in hydroxide ion concentration. It was also found that above pH 9 all reaction took place via initial formation of uridine 2′,3′-cyclic phosphorothioate without any significant competition by desulfurization. Alkaline cleavage of an oligoribonucleotide and its thio analogue also exhibited little difference in reactivity, and intermolecular reactions of methyl 2,4-dinitrophenyl phosphate with different nucleophiles gave so-called "thioeffects" ($k_{\text{phosphate}}/k_{\text{thioate}}$) of 4−11.[10] A more extensive study on the pH dependence of the

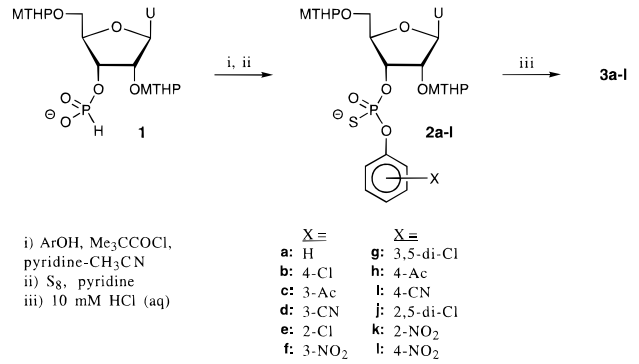
* To whom correspondence should be addressed at Laboratory of Organic and Bioorganic Chemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1996.

(1) Knowles, J. R. *Annu. Rev. Biochem.* **1980**, *49*, 877.
(2) Eckstein, F. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 423.
(3) Eckstein, F. *Annu. Rev. Biochem.* **1985**, *54*, 367.
(4) Eckstein, F.; Gish G. *TIBS* **1989**, *14*, 97.
(5) Frey, P. A. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1989**, *62*, 119.
(6) Wagner, R. W. *Nature* **1994**, *372*, 333.
(7) Uhlmann, E.; Peymann, A. *Chem. Rev.* **1990**, *90*, 543.
(8) Mizrahi, V.; Henrie R. N.; Marlier J: F.; Johnson K. A.; Bencovic, S. J. *Biochemistry* **1985**, *24*, 4010.
(9) McSwiggen, J. A.; Cech, T. R. *Science* **1989**, *244*, 679.
(10) Herschlag, D.; Piccirilli, J. A.; Cech, T. R. *Biochemistry* **1991**, *30*, 4844.
(11) Herschlag, D. *J. Am. Chem. Soc.* **1994**, *116*, 11631.
(12) Breslow, R.; Katz, I. *J. Am. Chem. Soc.* **1968**, *90*, 7376.
(13) Hollfelder, F.; Herschlag, D. *Biochemistry* **1995**, *34*, 12255.
(14) Ketelaar, J. A. A.; Gersmann, H. R.; Koopmans, K. *Recl. Trav. Chim. Phys-Bas* **1952**, *71*, 1253.
(15) Fanni, T.; Taira, K.; Gorenstein, D. G.; Vaidyanathaswany, R.; Verkade, J. G. *J. Am. Chem. Soc.* **1986**, *108*, 6311.
(16) Eckstein, F. *FEBS Lett.* **1968**, *2*, 85.
(17) Smrt, J.; Malkiewicz, A. *Collect. Czech. Chem. Commun.* **1973**, *38*, 2962.
(18) Burgers, P. M. J.; Eckstein, F. *Biochemistry* **1979**, *18*, 592.
(19) Almer, H.; Strömberg, R. *Tetrahedron Lett.* **1991**, *32*, 3723.

S0002-7863(95)03399-3 CCC: $12.00

Scheme 1



reactions of the R_P and S_P diastereoisomers of uridine 3′-(uridine 5′-phosphorothioate) has recently been carried out.[20] The rate of decomposition did not vary that much over most of the pH range and was essentially similar to that reported in the previous study on the same compounds (above and ref 19) except at strongly acidic conditions (pH < 1) where the thioates react at a 30-fold lower rate than the phosphate.[20] At this pH, the disappearance of the dimers occurs by three different routes of comparable rate (desulfurization, cleavage, and isomerization). It was also found that desulfurization was the predominant reaction at pH 3−6.

Aryl-containing phosphorothioates not only serve as tools with which reactions can be conveniently and accurately monitored (continously with UV spectroscopy) and as compounds for elucidation of more detailed information on reaction mechanisms, but are also of direct use as substrates when enzyme-catalyzed reactions are probed.[12,13] We have therefore chosen uridine 3′-(aryl phosphorothioates) in the present investigation on base-catalyzed intramolecular transesterification of phosphorothioate diesters. This also gives the added advantage that comparable data for the corresponding phosphodiesters are available.[21] The leaving group dependence for the hydroxide- and imidazole-catalyzed reactions as well as the dependence on the base has been investigated, and details of the mechanism are discussed and compared to those for phosphodiesters.

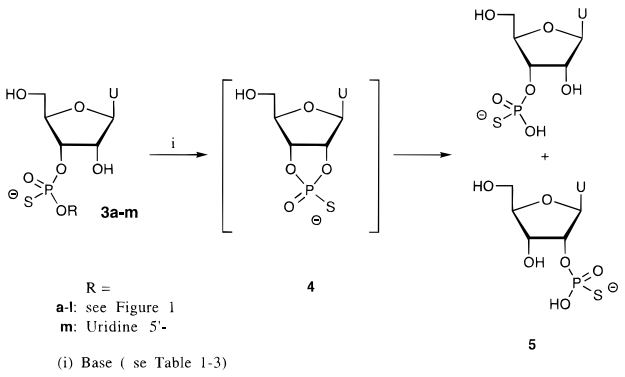
Results and Discussion

Synthesis of Uridine 3′-(Aryl phosphorothioate)s. The uridine (aryl phosphorothioate)s used in this study were synthesised using a novel method for synthesis of alkyl aryl phosphorothioates (Scheme 1). The method makes use of a convenient one-pot procedure based on the H-phosphonate approach[22−26] which has been used in, e.g., synthesis of dinucleoside phosphorothioates.[27−29] The readily available 2′,5′-bis(methoxytetrahydropyranyl)uridine 3′-H-phosphonate (**1**)[30] is condensed with the desired phenol using pivaloyl chloride, and the resulting H-phosphonate diester is then subsequently sulfurized with elemental sulfur to give the uridine 3′-(aryl phosphorothioate) **2**. Sulfurization is virtually quantitative, but the condensation reaction required some tuning. The lower nucleophilicity of phenols relative to alkanols should not pose any problem under H-phosphonate condensation conditions, but there was some question whether the presence of an aryl group in the H-phosphonate diester would inflict secondary reactions such as further activation to an acyl phosphite by the condensing agent. A dialkyl H-phosphonate is not activated in this way by excess coupling reagent.[22,31] Prolonged exposure to excess pivaloyl chloride can instead produce acyl phosphonates, although this is a rather slow process.[32,33] Esters of more acidic alcohols such as phenols could however have a different phosphonate−phosphite equilibrium and be more prone to subsequent reactions, leading to tricoordinated acyl phosphite derivatives, and one could expect this to be more pronounced with more acidic phenols. When coupling between the nucleoside H-phosphonate **1** and more acidic phenols was followed by ^{31}P NMR, we did indeed observe resonances in the 130−140 ppm range which is indicative of trivalent phosphorus species. However, by decreasing the amount of condensing agent, we minimized the undesirable reactions and could synthesize the uridine 3′-(aryl phosphorothioate)s in yields of 44−91% of isolated material. The kinetic studies below were done with the isomeric mixtures unless otherwise stated, and the isomeric ratio (always more of the S_P isomer) was determined from integration of ^{31}P NMR resonances. The configurational assignment was based on the relative ^{31}P chemical shifts (the protected R_P isomer of a dinucleotide always seems to resonate downfield of the S_P isomer whereas the opposite is found for the deprotected derivatives[2,27]), and the reported observations that H-phosphonate condensations with protected ribonucleoside H-phosphonates always seem to be stereoselective, forming more of the R_P isomers after sulfurization.[27−30] It should be noted that the S_P isomers of the uridine 3′-(aryl phosphorothioate)s have the same configuration as the R_P isomer of a dinucleotide but are denoted differently since the priority rules for the nomenclature demand this (the aryl substituents have higher priority than a nucleoside).

Reactions of Uridine 3′-(Aryl phosphorothioate)s. In order to be certain whether the pathways of the reactions were as expected or accompanied by other events, we monitored a number of reactions using ^{31}P NMR spectroscopy. When the removal of acetal groups (in 0.01 M HCl) from the phenyl (**2a**) and the 2-nitrophenyl (**2k**) derivatives was monitored, no evidence of side reaction was observed, only a slight shift of the ^{31}P resonances when **2** was converted to **3**. Furthermore, the reactions of **3a**, **3e**, and **3k** as well as of the R_P and S_P diastereoisomers of uridine 3′-(uridine 5′-phosphorothioate) (**3m**) were monitored under several different conditions similar to those used in the kinetic measurements, imidazole buffer (pH 7.06), carbonate buffer (pH 9.37), and 0.01 M sodium hydroxide (**3m**). In none of the reactions could we observe any competing desulfurization, not even at pH 7 where this is observed for **3m**.[20] This is not unexpected since an aryloxy leaving group is considerably better than the 5′-oxyanion in a dinucleotide and departure of SH^- cannot compete with the transesterification. As can be expected with a leaving group that is

(20) Oivanen, M.; Ora, M.; Almer, H.; Strömberg, R.; Lönnberg H. *J. Org. Chem.* **1995**, *60*, 5620.
(21) Davis, A. M.; Hall, A. D.; Williams, A. *J. Am. Chem. Soc.* **1988**, *110*, 5105.
(22) Garegg, P. J.; Regberg, T.; Stawinski, J.; Strömberg, R. *Chem. Scr.* **1985**, *25*, 280.
(23) Garegg, P. J.; Regberg, T.; Stawinski, J.; Strömberg, R. *Chem. Scr.* **1986**, *26*, 59.
(24) Froehler, B. C.; Matteucci, M. D. *Tetrahedron Lett.* **1986**, *27*, 469.
(25) Strömberg, R. *Chem. Commun. Stockholm Univ.* **1987**, 1.
(26) Stawinski, J.; Strömberg, R. *Trends Org. Chem.* **1993**, *4*, 31.
(27) Almer, H.; Stawinski, J.; Strömberg, R.; Thelin, M. *J. Org. Chem.* **1992**, *57*, 6163.
(28) Seela, F.; Kretschmer, U. *J. Org. Chem.* **1991**, *56*, 3861.
(29) Battistini, C.; Brasca, M. G.; Fustinoni, S.; Lazzari, E. *Tetrahedron* **1992**, *48*, 3209.
(30) Szabó, T.; Almer, H.; Strömberg, R.; Stawinski, J. *Nucleosides Nucleotides* **1995**, *14*, 715.
(31) Garegg, P. J.; Regberg, T.; Stawinski, J.; Strömberg, R. *Nucleosides Nucleotides* **1987**, *6*, 655.
(32) Kuyl-Yeheskiely, E.; Spierenberg, M.; van den Elst, H.; Tromp, M.; van der Marel, G. A.; van Boom, J. H. *Recl. Trav. Chim. Pays-Bas* **1986**, *105*, 505.
(33) Strömberg, R.; Stawinski, J. *Nucleic Acids Res. Symp. Ser.* **1987**, *18*, 185.

Scheme 2



considerably better than the 3′-oxyanion, no isomerization to the 2′,5′-linked diester could be detected. The reaction proceeded cleanly through the uridine 2′,3′-cyclic phosphorothioate (**4**) (^{31}P NMR: $\delta = 74.7-75.2$ ppm (S_P) and 76.2−76.6 ppm (R_P) depending on conditions) as the only observed pathway (Scheme 2). When using the separated isomers of **3m**, we could also observe that the reaction proceeds by complete inversion of configuration (i.e., it is stereospecific). With the aryl derivatives the cyclization step was virtually complete before much of the subsequent hydrolysis to the uridine 2′- and 3′-phosphorothioates **5** had occurred. With **3m** the two reaction steps were of comparable rates so that **3**, **4**, and **5** coexisted in the reaction mixture in substantial quantities and throughout most of the time needed for complete hydrolysis.

The pseudo-first-order rate constants for the different aryl esters were obtained by measuring the release of phenolate anions with time. All reactions showed excellent first-order kinetics up to 90% of the total reaction. The absence of any substantial deviation, which can occur due to parallel reactions of two compounds, forming a common product,[34,35] pointed toward a rather small difference in rate constants for the isomers. The diastereoisomers of the compounds at the extremes of the pK_a (ArOH) values, i.e., the nitrophenyl and phenyl derivatives, could be separated after repeated HPLC purification. The rate constants for the separate isomers were determined in different buffers over most of the pH range used and were indeed found to be rather similar, $k_{R_P}/k_{S_P} = 1.4 \pm 0.04$ under all conditions employed. This is similar to what is found for the base-catalyzed reaction of the dinucleoside phosphorothioate **3m** where the thioate having the same spatial arrangement of phosphorus ligands also reacts faster, i.e., $k_{S_P}/k_{R_P} = 1.7$.[19,20] Because of the small and seemingly constant ratio of the rate constants for the different isomers, we employed simple first-order analysis of the kinetics. The rate constants for the S_P isomers were calculated using a constant for each compound that was derived from the isomeric composition (always more S_P) and a first-order curve fit to a simulated plot of ln(fraction of product) with time, derived from equations for two parallel reactions with relative rate constants as for our isomers. Direct use of the experimental data from the isomeric mixtures without separating out the respective rate constants gave slopes of the Brønsted plots within the error values obtained when the data for one isomer were plotted.

The hydroxide ion catalyzed reactions of the substituted uridine aryl phosphorothioates were followed at different pH values in hydrogen carbonate/carbonate buffers (at constant ionic strength). The second-order rate constants for the hydroxide ion catalyzed reaction, k_{OH}, were determined from the slopes of the plots of the pseudo-first-order rate constants k' versus the hydroxide ion concentrations. The results are listed in Table 1.

Table 1. Intramolecular Transesterification in Uridine 3′-(Aryl (S_P)-phosphorothioate)s Catalyzed by Hydroxide Ion at 25 °C and 0.25 M Ionic Strength (Maintained with Na_2SO_4)[a]

substituent	pK_a(ArOH)	k_{OH}/(M^{-1} s^{-1})	pH	$k' \times 10^4/s^{-1}$	λ/nm
H	9.95	7.0	9.1−11	1.4−30	230
4-Cl	9.38	22	8.7−11	2.7−76	238
3-Ac	9.19	24	8.9−10	2.9−29	233
3-CN	8.61	68	8.9−10	11−84	318
3-NO_2	8.35	101	8.5−10	8.3−130	238
3,5-di-Cl	8.18	163	8.7−10	19−130	297
4-Ac	8.05	77	8.5−10	5.2−86	325
4-CN	7.95	122	8.5−10	8.3−150	266
2,5-di-Cl	7.51	155	8.7−10	13−100	299
2-NO_2	7.23	304	8.7−10	29−240	415
4-NO_2	7.14	295	8.7−10	34−620	400

[a] The substrate concentration was about 0.1 mM. The values for the S_P isomers are tabulated; the rate constants for the R_P isomers are 1.4 times higher. The range of pH used was accomplished with 0.05 M bicarbonate/carbonate buffers to give the range of observed rate constants. The number of data points (not including duplicates) is 6−10 for each compound. Wavelengths given are those used for monitoring the kinetics. It should be noted that the S_P isomer of the uridine 3′-(aryl phosphorothioate)s has the same configuration as the R_P isomer of a dinucleotide.

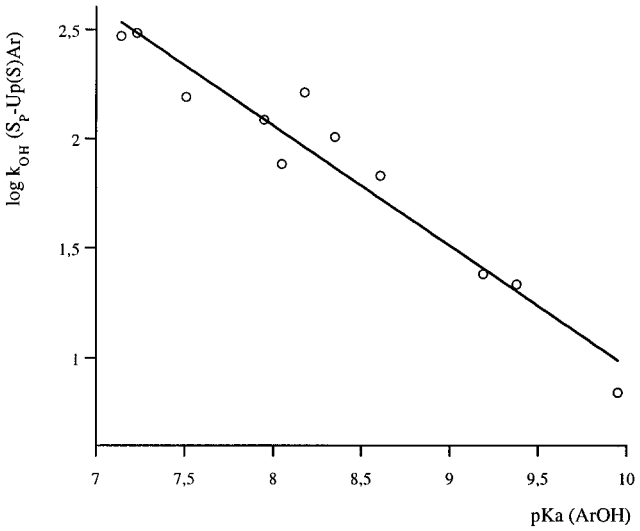


Figure 1. Brønsted linear free energy relationship of the leaving group dependence of the fission of uridine 3′-(aryl (S_P)-phosphorothioate)s as catalyzed by hydroxide ion at 25 °C. Linear regression gives a calculated line (eq 1) with a slope that corresponds to a β_{lg} of −0.55. The corresponding evaluation for the R_P isomers creates a parallel line 0.15 log unit higher.

The second-order rate constants for hydroxide ion catalyzed reactions (k_{OH}) were found to obey a Brønsted linear free energy relationship with respect to the acidity of the conjugate acid of the leaving phenol (Figure 1). The equation for the linear curve fit (eq 1) gives a $\beta_{\text{leaving group}}$ (β_{lg}) value of −0.55 for the

$$\log k_{OH} = (-0.55 \pm 0.05)pK_a(ArOH) + 6.4 \pm 0.4 \quad (1)$$

hydroxide ion catalyzed reaction of the uridine 3′-(aryl phosphorothioate)s. This value is quite comparable to the $\beta_{lg} = -0.54$ found for the hydroxide ion catalyzed reaction in the corresponding uridine 3′-(aryl phosphate)s.[21]

The intramolecular transesterification of the uridine aryl phosphorothioates appears to exhibit general base catalysis (Table 2). The kinetically equivalent alternative, specific base−general acid catalysis, is less likely since we have a relatively

(34) Brown, H. C.; Fletcher R. S., *J. Am. Chem. Soc.* **1949**, *71*, 1845.
(35) Moore, J. W.; Pearson, R. G. *Kinetics and Mechanism*, 3rd ed.; John Wiley & Sons: New York and Chichester, 1981.

Table 2. Intramolecular Transesterification in Uridine 3′-(4-Nitrophenyl (S_P)-phosphorothioate) Catalyzed by Nitrogen Bases at 25 °C and 0.25 M Ionic Strength (Maintained with Na_2SO_4)[a]

base[a]	pK_a (BH)	$k_B \times 10^3/(M^{-1}\,s^{-1})$	pH
β-alanine	10.19	125	9.0
glycine	9.60	61	8.8
Tris[d]	8.10	6.3	8.2
imidazole	6.95	1.4	7.0

[a] The wavelength used for kinetics was 400 nm. The buffer concentration ranged from 0.1 to 0.5 M; the substrate concentration was 0.1 mM. The rate constants (k_B) presented are for the S_P isomers; the values for the R_P isomers are 1.4 times higher. It should be noted that the S_P isomer of the uridine 3′-(aryl phosphorothioate)s has the same configuration as the R_P isomer of a dinucleotide.

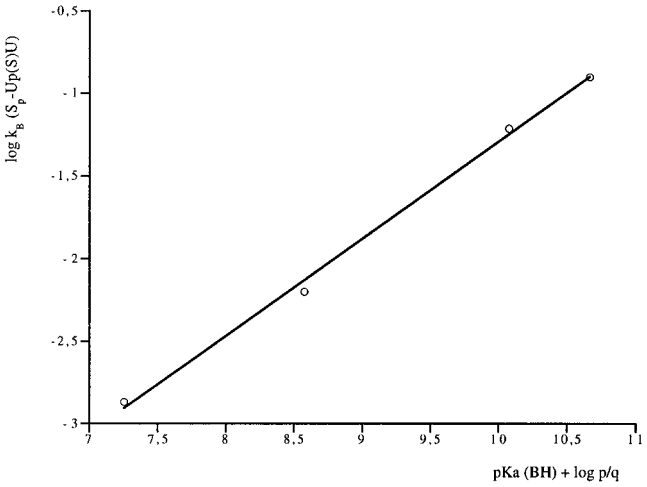


Figure 2. Brønsted dependence of the fission of uridine 3′-(4-nitrophenyl S_P-phosphorothioate) as catalyzed by general bases at 25 °C. Statistical correction by adding log(p/q) to the pK_a(BH) is employed (Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover Publishers: New York, 1987) where p is the number of equivalent protons on the acid and q is the number of equivalent positions where a proton can be accepted in the conjugate base). The slope of the calculated line (eq 2) gives a β value of 0.59. The corresponding evaluation for the R_P isomers creates a parallel line 0.15 log unit higher.

good leaving group. It has been concluded that general base catalysis occurs in a similar reaction of a uridine phosphate derivative with the even poorer leaving group (4-nitrophenoxy)-methanol.[36] The observed rate constants are linearly dependent on the concentration of the buffers at constant pH without sign of deviation (the value for the imidazole-catalyzed reaction is however obtained as discussed below). The equation for the linear curve fit of the plot of k_B against log pKa + log(p/q) (Figure 2) for the conjugate acid of the base (eq 2) gives a Brønsted β value of 0.59.

$$\log k_B = (0.59 \pm 0.02)(pK_a(BH) + \log(p/q)) - 7.2 \pm 0.2 \quad (2)$$

The second-order rate constants for imidazole-catalyzed (k_{Im}, Table 3) cleavage were also found to obey a Brønsted linear free energy relationship with respect to the acidity of the leaving phenol (log $k_{Im-I} = (-0.64 \pm 0.07)pK_a(ArOH) + 1.5 \pm 0.5$). Marriot and Kirby[36] have however shown that an increase of buffer concentration can give a medium effect due to the increase in free imidazole. With this brought to our attention, we noticed that there is a similar tendency also in the imidazole-catalyzed reactions of the uridine 3′-(aryl phosphorothioate)s. In order to keep this nonionic medium effect under control, we

(36) Kirby, A. J.; Marriott, R. E. *J. Am. Chem. Soc.* **1995**, *117*, 833.

Table 3. Intramolecular Transesterification in Uridine 3′-(Aryl (S_P)-phosphorothioate)s Catalyzed by Imidazole at 25 °C and 0.25 M Ionic Strength (Maintained with Na_2SO_4)[a]

substituent	pK_a(ArOH)	$k_{Im-I} \times 10^4/(M^{-1}\,s^{-1})$	$k_{Im-IO} \times 10^4/(M^{-1}\,s^{-1})$ (with MeCN)
3-CN	8.61	1.1	1.6
3-NO_2	8.35	1.7	2.6
4-Ac	8.05	1.9	2.8
4-CN	7.95	2.8	4.4
2,5-di-Cl	7.51	4.3	6.7
2-NO_2	7.23	6.7	13
4-NO_2	7.14	13	14

[a] Column three (k_{Im}) shows the values obtained when only the ionic strength is maintained and column four (k_{Im} (with MeCN)) the values obtained when also the molarity of the nonionic organic material is maintained (with acetonitrile). Wavelengths used for the kinetic measurements with the respective compounds are the same as in Table 1. The buffer concentration was 0.1−0.5 M with 50% or 95% free imidazole. The values for the S_P isomers are tabulated; the rate constants for the R_P isomers are 1.4 times higher. It should be noted that the S_P isomer of the uridine 3′-(aryl phosphorothioate)s has the same configuration as the R_P isomer of a dinucleotide.

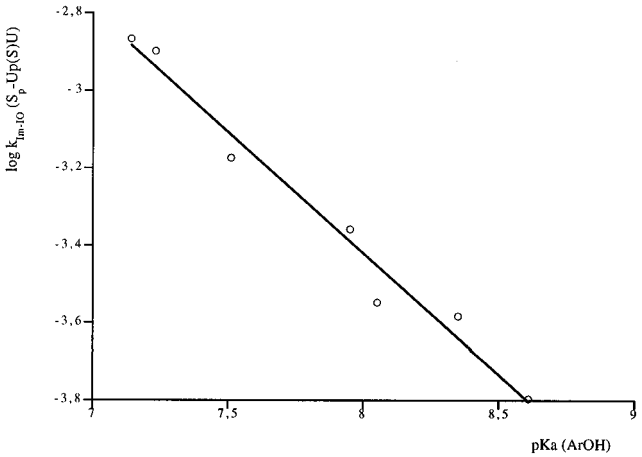


Figure 3. Brønsted linear free energy relationship of the leaving group dependence of the fission of uridine 3′-(aryl (S_P)-phosphorothioate)s as catalyzed by imidazole at 25 °C. Linear regression gives a calculated line (eq 3) with a slope that corresponds to a β_{lg} of −0.63. The corresponding evaluation for the R_P isomers creates a parallel line 0.15 log unit higher.

used acetonitrile (chosen because the dipole moment is similar to that of imidazole, 3.8 and 3.9 D, respectively) to compensate for free imidazole when the buffer concentration is varied (i.e., keeping the molarity of the organic component constant). With this procedure, a better correlation of the linear dependence upon buffer concentration was obtained and the values of the second-order rate constants were about 50−80% higher for most uridine 3′-(aryl phosphorothioate)s (k_{Im-IO} (with MeCN), Table 3). A better linear correlation was also obtained for the Brønsted plot when these values were used (Figure 3). The β_{lg} value obtained is however virtually identical to that from the k_{Im-I} values (i.e., when only the ionic strength is kept constant). The linear curve fit of the Brønsted plot (log k_{Im-IO} *vs* pK_a(ArOH)) for the imidazole-catalyzed reaction of the uridine 3′-(aryl phosphorothioates) gives a β_{lg} value of −0.63 (eq 3).

$$\log k_{Im-IO} = (-0.63 \pm 0.04)pK_a(ArOH) + 1.6 \pm 0.3 \quad (3)$$

Phosphorothioate Diesters *vs* Phosphodiesters. The thio effect derived from the equations of the Brønsted plots of the hydroxide ion-catalyzed reactions of uridine 3′-(aryl (S_P)-phosphorothioate)s and the uridine 3′-(aryl phosphates) of Davis *et al.*[21] is that $k_{OH(UpAr)}/k_{OH(S_P-Up(S)Ar)} = 2.4-2.5$ and for the R_P

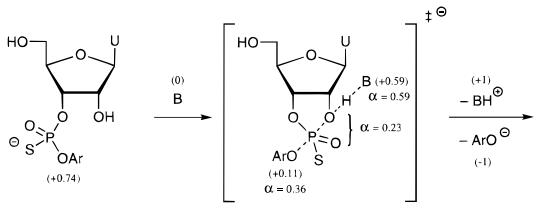
isomer the corresponding value is 1.7−1.8. These values are slightly higher than for base-catalyzed hydrolysis in a diribonucleotide, **3m** ($k_{(O)}/k_{R_P(S)} = 1.3$ and $k_{(O)}/k_{S_P(S)} = 0.78$)[17−19] in the direction of that for triesters ($k_{(O)}/k_{(S)} \approx 10-100$)[14,15] and acid-catalyzed cleavage of **3m** ($k_{(O)}/k_{(S)} \approx 2-10$ at pH 1−2)[20] which could be expected when the reactivity of a diester is increased.

When the equations of the Brønsted plots are compared for sensitivity to the leaving group in the imidazole-catalyzed reactions (k_{Im-I} to be able to compare directly with the data of Davis *et al.* [21]) and for sensitivity to the base used, differences of a similar order are obtained for the ratio of the phosphate and (S_P)-phosphorothioate ($k_{Im(UpAr)}/k_{Im(S_P-Up(S)Ar)} = 1.7-2.1$ and $k_{B(UpAr)}/k_{B(S_P-Up(S)Ar)} = 1.9-3.6$). What can be said about the thio effects is that uridine 3′-(aryl (S_P)-phosphorothioate)s are less reactive than the corresponding phosphates by a factor of about 2 and under some conditions up to about 4. For the R_P isomer the thio effect ranges from 1.2 to 2.6. The difference in reactivity for the thioates and phosphates is relatively insensitive to which catalyst is being used or which group that leaves, in particular the latter. This can also be concluded from the relatively small difference when reactions of the aryl esters **3** are compared with those of dinucleotides[17−20] despite the large difference in leaving group ability.

It seems that the reaction mechanism for intramolecular transesterification in uridine 3′-aryl or alkyl phosphorothioates must be rather similar to that for the corresponding phosphate esters even in details such as the degree of bond breaking and making in the transition state. A small difference can be observed though; the Brønsted β value of 0.59 for dependence on the basicity of the catalyst is a bit lower than the value of 0.67 found for uridine 3′-(aryl phosphates)[21] whereas the β_{lg} for the imidazole-catalyzed reaction is slightly higher (−0.63 or −0.64 compared to −0.59). The β_{lg} value was not much affected by the medium effect of imidazole, and considering the similarity in mechanism, it is likely that this would also be the case for the uridine 3′-(aryl phosphates) used by Davis *et al.* A small effect would presumably be in the same direction as for the thioates. The reaction mechanism may be quite similar for phosphates and phosphorothioates, but it does not seem obvious that the reaction with the sulfur analogue should be slower. An oxygen is more electronegative, but the effect from that could be overruled by the greater polarizibility of sulfur, thus rendering the thio analogue more able to carry negative charge (cf. pK_a values of thiols and alcohols). Spectroscopic data also suggest that most of the charge in a phosphorothioate anion resides on sulfur.[37]

Relative rates of reaction with external nucleophiles of triesters and the corresponding diesters depend strongly on the charge on the nucleophile. In displacement of 2,4-dinitrophenolate from a triester or corresponding diester, the ratio is less than 100 for neutral nucleophiles (26 for water, 2−40 for pyridines), but several thousand for anions (4000 for phosphate, nearly 5000 for fluoride).[38] This suggests that the electrostatic repulsion is a most important factor in nucleophilic attack toward phosphate centers. Since the charge can be more easily dispersed with a polarizable sulfur present, one could expect less electrostatic repulsion with phosphorothioates than with phosphates. The thioeffect for nucleophilic attack on methyl 2,4-dinitrophenyl phosphate and phosphorothioate is dependent on the charge of the nucleophile (OH^- and F^- give $k_{(O)}/k_{(S)} =$ 4.1 and 4.5 whereas the thio effect for formate ion is 9 and for pyridine 11)[10] which suggests that electrostatic repulsion is less important for a phosphorothioate than for a phosphate ester. That the difference in reactivity in the studied transesterifications is opposite to what one could expect from the above arguments can perhaps, at least partially, be explained by solvation of the transition state. One could expect this to be energetically more favorable with a phosphate ester since hydrogen bonding is stronger when the charge density is higher; solvation would then counteract the other effects mentioned above. If solvation is of prime importance then this could perhaps also explain why phosphorothioate triesters are hydrolyzed at a considerably lower rate (about 10−100 times slower)[14,15] than their phosphate counterparts. The transition state for the rate-limiting step in hydrolysis of triesters probably resembles an intermediate phosphorane anion, and the sulfur in a thioate would then carry the charge whereas an oxygen does so in the ground state (OH^-). It seems feasible that the transition state for the sulfur analogue is less stabilized (relative to the ground state) by solvation than that for the oxygen counterpart.

Scheme 3



Reaction Mechanism. Williams *et al.*[21] have analyzed the transition state for phosphate transfer in uridine 3′-(aryl phosphates) by using an effective charge approach[39,40] to obtain Leffler α values.[41] Irregularities have in some cases been observed in these kinds of analyses[42,43] which makes proper treatment of these relationships essential to give useful effective charge data. Williams has argued convincingly for the validity and usefulness of the effective charge approach in numerous phosphate transfer reactions,[21,39,40,44] and this should apply equally well with thio analogues, especially when used for comparison with phosphates. His analysis of uridine 3′-(aryl phosphate)s[21] gave an imbalance in the Leffler α values of 0.33 which is suggestive of a transition state with charge buildup on the attacking oxygen and/or phosphate function. A similar kind of analysis for the phosphorothioate analogues in the present study can also be done if we assume that the effective charge on the aryloxy oxygen of the uridine aryl phosphorothioates is not too different from that on the phosphate counterparts (or rather $ArOPO_3H^-$)[42] which is +0.73 to 0.74. This does not seem unreasonable since the effective charge on the ether oxygen in esters is relatively insensitive to substituents (in the acid part) when the formal charge is not changed,[39] an example being the similarity between the bridging oxygens in the above-mentioned phosphates and aryl sulfates ($ArOSO_3^-$), the latter having an effective charge of +0.7.[45] With a value of 0.74 for the effective charge on the aryloxy oxygen, a β of 0.59 for the dependence on basicity, and a β_{lg} of −0.63 for the imidazole-catalyzed reaction, we arrive at Leffler α values of 0.59 for proton abstraction and 0.36 for bond breaking to the leaving group in the transition state (Scheme 3) which leaves an imbalance in the α values of 0.23. This indicates that some negative charge is built up on the central group of atoms, but

(37) Frey, P. A,; Sammons, R. D. *Science* **1985**, *228*, 541.
(38) Kirby, A. J.; Younas, M. *J. Chem. Soc., Sect. B* **1970**, 1165.
(39) Thea, S.; Williams, A. *Chem. Soc. Rev.* **1986**, *15*, 125.
(40) Williams, A. *Acc. Chem. Res.* **1984**, *17*, 425.
(41) Leffler, J. E. *Science* **1953**, *117*, 340.
(42) Bordwell, F. G.; Boyle, W. J. *J. Am. Chem. Soc.* **1972**, *94*, 3907.
(43) Pross, A. *Tetrahedron Lett.* **1983**, 835
(44) Bourne, N.; Williams, A. *J. Org. Chem.* **1984**, 1200 (1984).
(45) Hopkins, A.; Day, R. A.; Williams, A. *J. Am. Chem. Soc.* **1983**, *105*, 6062.

we cannot distinguish between the possibilities of where most of the charge density is concentrated.

The intramolecular transesterification in uridine 3′-(aryl phosphorothioates) is an associative process with a mechanism that is similar to that of the corresponding phosphate esters. It has been argued persuasively that nucleophilic displacement of a good leaving group from a phosphate by oxyanions either is a concerted reaction or involves an intermediate in a very shallow energy well at the top of a barrier.[21] The slight difference found between phosphates and their thio analogues suggests this is so also for phosphorothioates. The simultaneous dependence of base and leaving group is also consistent with a concerted process. When the details of the reactions are compared, it seems that slightly less charge is built up in the reactions of the phosphorothioates ($\alpha = 0.23$) compared to those of the corresponding phosphates ($\alpha = 0.33$). This is indicative of a slightly more dissociative character of the transition state for the base-catalyzed transesterification in uridine 3′-(aryl phosphorothioates) than for the same reaction in uridine 3′-(aryl phosphate)s. A slightly higher degree of bond breakage to the leaving group in the transition state ($\beta_{lg} = -0.63$ compared to -0.59) points in the same direction. This is also consistent with the higher ability of thiophosphate monoesters in undergoing reactions via a dissociative mechanism with intermediate formation of a thiometaphosphate.[12,46−48]

The difference in cleavage rate for phosphodiesters and phosphorothioate diesters is rather small. The well-known greater stability of phosphorothioate analogues of oligonucleotides for use in therapeutic applications[2,3,6,7] can thus not be due to their greater inherent chemical stability but is rather due to resistance toward enzymatic degradation. Phosphorothioate analogues of DNA fragments used for therapeutics do however have a limited life time,[6,7] and one could consider this to be due to oxidative desulfurization. However, that this would occur by action of molecular oxygen is less likely in view of this and previous studies[19,20] since this pathway could not be detected. Action of nucleases or metal ions or oxidation by biomolecules seems like a more probable cause.

We believe our investigation has provided some insight into the details of reaction mechanisms for transesterification of phosphorothioate diesters and their relation to mechanisms for phosphodiesters. The study also provides a basis for use of these specific and similar compounds in detailed studies of mechanisms of enzyme-catalyzed phosphate transfer reactions. An example of this kind of usage for monoesters can be found in studies of alkaline phosphatase where β_{lg} values for phosphate and phosphorothioates have been used to try to elucidate whether an associative or dissociative process takes place in the active site of the enzyme.[12,13,49] It is evident from the similarity in mechanism that phosphorothioates can be most suitable for studies of enzyme mechanisms in vitro and possibly also for in vivo studies. If substantial differences in the cleavage rate of biologically active phosphates and their phosphorothioate analogues are found, this cannot be due to their respective inherent reactivities but would rather suggest some specific interaction with the nonbridging oxygen/sulfur, e.g., hydrogen bonding or interaction with a metal ion.

Experimental Section

Materials. Pyridine (Merck pa), acetonitrile (Merck pa), and triethylamine (Aldrich) were dried by refluxing over CaH_2, distilled, and stored over molecular sieves or CaH_2 (triethylamine). Pivaloyl chloride was distilled at atmospheric pressure and stored at −20 °C in sealed flasks. Chloroform was passed through basic Al_2O_3 prior to use. Uridine (Sigma), toluenesulfonic acid monohydrate (Fluka), trimethyl orthoacetate (Aldrich), 4-methoxy-5,6-dihydro-2*H*-pyran (Aldrich), elemental sulfur (Aldrich), imidazole (Fluka pa), glycine (Merck pa), tris(hydroxymethyl)aminomethane (Merck pa), β-alanine (Janssen), sodium carbonate (Merck pa), sodium hydrogen carbonate (Merck pa), sodium sulfate (Merck pa), and the phenols (Aldrich) were all commercial grade. Water used for buffers was doubly distilled from a glass apparatus (acid washed every six months).

Methods. ^{1}H and ^{31}P NMR spectra were recorded on a Jeol GSX-270 FT spectrometer with samples in 5 or 10 mm NMR tubes. ^{31}P chemical shifts are given relative to 2% H_3PO_4 in D_2O as external reference (in a coaxial inner tube), and ^{1}H chemical shifts are relative to tetramethylsilane. High-resolution FAB mass spectra were recorded on a Jeol SX-102 instrument. The pH of the buffers was determined using a Radiometer pHM 62 standard pH meter instrument calibrated to ±0.01 pH unit with standard buffers.

Reactions Monitored by ^{31}P NMR. The studies for optimization of the synthetic route by looking at the effect of varying the amount of pivaloyl chloride in the H-phosphonate condensation were performed as follows: The appropriate phenol (60 μmol) and **1** (32 mg, 50 μmol) were rendered anhydrous by evaporation of added pyridine and dissolved in pyridine (2 mL). Pivaloyl chloride (75−200 μmol) was added and the reaction monitored with time.

Studies of deprotection of the methoxytetrahydropyranyl protection was done by dissolving the protected diester (10 μmol) in 0.01 M HCl (0.1 mL), and after 1 h H_2O (0.4 mL) was added. The stock solution was stored at −18 °C and monitored once a week for three weeks.

For the reactions in carbonate buffer the protected diester (10 μmol) was first dissolved in 0.01 M HCl (0.1 mL), and after 2 h sodium carbonate/bicarbonate buffer (0.067 M, 0.3 mL) was added, resulting in pH 9.37. The course of the reaction was then monitored with time at this pH and 25 °C. The reactions in imidazole buffer were carried out in a similar way. The protected diester (10 μmol) was dissolved in 0.01 M HCl (0.1 mL), and after 2 h 0.5 M imidazole buffer (0.3 mL) was added, resulting in pH 7.06, whereupon monitoring of reactions began.

The hydroxide-catalyzed reactions of the R_P and S_P isomers of **3m** were carried out in the following way: The dinucleotide (50 μmol) was dissolved in 0.01 M NaOH in water−dioxane (2 mL, 1:2 v/v) in a 10 mm NMR, tube and the course of reaction with time was followed by ^{31}P NMR.

Kinetic Methods. The substrates were stored as their 2′,5′-*O*-bis-(methoxytetrahydropyranyl) derivatives, and these where deprotected as required by the following technique. The protected aryl nucleoside phosphorothioate (10 μmol) was dissolved in 0.01 M HCl (200 μL), and after 2 h, water (800 μL) was added. These stock solutions could be used for several weeks. The stability of the deprotected compounds when stored at −18 °C in these acidic solutions was controlled with ^{31}P NMR, and there was no sign of desulfurization or other decomposition after three weeks of storage.

For a typical kinetic run, an aliquot (0.8 μL) of the above solution was added into a 1.5 mL quartz cell containing the appropriate buffer (1 mL). The reactions were monitored by following the time dependences of UV absorption (A_t) using a Varian Cary 3 dual-beam UV−vis spectrophotometer. Repetetive wavelength scanning during the reaction indicated the best single fixed wavelength to use for each compound in the kinetic studies. The wavelengths employed are given in Table 1. A thermostated cell block was used (25.0 ± 0.2 °C) and controlled by the Cary temperature controller. The reactions were followed until completion of reaction was achieved, and the end point was determined by calculating an average absorbance value from the region where UV absorbance was virtually constant.

The release of phenolate anion *vs* time exhibited excellent pseudo-first-order kinetics up to 90% of the total reaction without any noticeable deviation. Pseudo-first-order rate constants k' were derived from the slope of the plot of $\ln(A_\infty - A_t)$ *vs* reaction time, where A_∞ is the end point absorbance and A_t is the absorbance at each specific time. Most kinetic runs were of isomeric mixtures isolated after synthesis. Since the ratio of rate constants for the separate isomers at both extremes of the pK_a(ArOH) and in six different buffers over the pH range 8.5−11

(46) Cullis, P. M.; Iagrossi, A. *J. Am. Chem. Soc.* **1986**, *108*, 7870.
(47) Cullis, P. M.; Misra, R.; Wilkins, D. J. *J. Chem. Soc., Chem. Commun.* **1987**, 1594.
(48) Harnett, S. P.; Lowe, G. *J. Chem. Soc., Chem. Commun.* **1987**, 1416.
(49) Hall, A. D.; Williams, A. *Biochemistry* **1979**, *25*, 4784.

was found to be invariable, $k_{R_P}/k_{S_P} = 1.4 \pm 0.04$, it seems reasonable to make the approximation that this is the case for all experiments (even the ratio of rates for phosphates and phosphorothiates does not vary much under the different conditions employed). Any small variation should only introduce minor errors. Because of the small difference in rate constants between the isomers, we employed simple first-order analysis of the kinetics. The isomeric composition was different for each substrate as determined by integration of ^{31}P NMR spectra. Using an average value, not taking into account the slightly different compositions, would introduce some error, so the simulation (see below) to obtain a conversion factor was done separately for each substrate.

The equation $\ln(1 - F_{(ArOH)}) = \ln(F_{(S_P)}e^{-1.0t} + F_{(R_P)}e^{-1.4t})$ was used to simulate the straight line one would obtain for a first-order fit to two parallel reactions with relative rate constants of 1.0 and 1.4, with $F_{(S_P)}$ being the fraction of S_P isomer and $F_{(R_P)}$ the fraction of R_P isomer. The equation $F_{(ArOH)} = 1 - (F_{(S_P)}e^{-1.0t} + F_{(R_P)}e^{-1.4t})$ was used to calculate the fraction of product formed to obtain the time for 90% conversion (as in the measured data) which was then used as the upper limit for the first-order fit of the simulated line. Equal time intervals were also employed in the simulation since this is the mode by which sampling takes place during kinetic runs. Linear regression analysis to the simulated line gave correlation coefficients in excess of what usually can be expected with experimental error present ($R > 0.9999$), thus implicating that the employment of a constant to recalculate the data to the rate for the respective isomer introduces little error.

The rate constants for the S_P isomers were obtained by dividing the observed experimental rate constant with a conversion factor that is equal to the slope obtained from linear regression of the simulated line for the parallel reactions of both isomers. The factors used are as follows: **3a** (H), 1.148; **3b** (4-Cl), 1.118; **3j** (2,5-di-Cl), 1.137; **3g** (3,5-di-Cl), 1.135; **3i** (4-CN), 1.154; **3f** (3-NO_2), 1.150; **3h** (4-Ac), 1.110; **3d** (3-CN), 1.113; **3c** (3-Ac), 1.090; **3k** (2-NO_2), 1.111; **3l** (4-NO_2), 1.153. The rate constants for the R_P isomers are consequently 1.4 times higher than for the S_P isomer.

The second-order rate constants k_{OH} were derived from the slope of the plot of the observed first-order rate constants (k') versus hydroxide ion concentration. The pH variation was achieved with 50 mM $NaHCO_3/Na_2CO_3$ buffer with ionic strength 0.25 M maintained with Na_2SO_4. The pH was varied between 8.50 and 10.63, and the phenol esters used are listed in Table 1.

The second-order rate constants k_B were derived from the slope of the plot of the first-order rate constants for the nitrophenyl ester **3l** versus the concentration of different nitrogen bases. The 4-nitrophenyl ester was used. The bases used and the pH of the buffers are listed in Table 2. The buffer concentration ranged from 0.1 to 0.5 M, the ionic strength of 0.25 M was maintained with Na_2SO_4, and the pH was adjusted with HCl or NaOH.

The first set of second-order rate constants for the imidazole-catalyzed reactions (k_{Im-I}) were derived from the slope of the plot of the first-order rate constants versus imidazole concentration (i.e., as free base). Buffer concentrations were varied between 0.1 and 0.5 M and contained 50% or 95% free imidazole. The second set of second-order rate constants for the imidazole-catalyzed reactions (k_{Im-IO} (with MeCN)) was obtained in an identical fashion with the difference that the molarity of nonionic organic material was maintained with acetonitrile. The ionic strength of 0.25 M was in both cases maintained with Na_2SO_4. The phenol esters used in these experiments are listed in Table 3.

Methods for Synthesis. General Procedure for Synthesis of the 2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(Aryl phosphorothioate) Triethylammonium Salts (2a-l). The appropriate phenol (84 μmol) and 2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-H-phosphonate triethylammonium salt[30] (**1**; 45 mg, 70 μmol) was dried by evaporation of added pyridine and then dissolved in pyridine (1 mL) and stirred. Pivaloyl chloride (140 μmol, 17 μL) was added, and after 20 min TLC indicated complete reaction. The sulfurization was made *in situ* by subsequent addition of sulfur (350 μmol, 11 mg). The sulfurization was complete after 2 h as shown by TLC. Pyridine (3 mL) and triethylamine (1 mL) were added, and the reaction mixture was evaporated to dryness. The reaction was dissolved in acetonitrile, the unreacted sulfur was filtered off, and the solvent was removed by evaporation. The product was purified on a silica gel column using a stepwise gradient of methanol (0−16%) in chloroform−triethylamine, 99:1 (v/v). The product was isolated as a diastereoisomeric mixture, and the ratio of each diastereomer was estimated with ^{31}P NMR or reversed phase HPLC (**2l**). The isomers of **2a** could be separated by repeated HPLC purification on a Rainin Dynamax silica gel column (10 × 250 mm) with a 60 min gradient of 0−5% MeOH in chloroform. The isomers of **2l** could be separated by repeated HPLC purification on a Merck Hibar Lichrosorb RP-18 column (7 μm, 25 × 250 mm) using an 80 min gradient of 0−50% MeCN in 0.1 mM triethylammonium acetate buffer (pH 6.2).

Phenyl 2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-Phosphorothioate Triethylammonium Salt (2a). Yield: 50 mg (96%). ^{31}P NMR (in pyridine): $\delta = 52.4$ (0.57 P, S_P isomer) and 52.2 (0.43 P, R_P isomer); HRMS: found (M^-) 643.1707, $C_{27}H_{36}N_2O_{12}SP$ requires M, 643.1727.

***S*$_P$ Isomer.** ^{1}H NMR (in D_2O): $\delta = 7.85$ (d, $^3J = 8.4$ Hz, 1 H, 6-H), 7.44−7.16 (m, 5 H, aromatic protons), 6.19 (d, $^3J = 7.7$ Hz, 1 H, 1′-H), 5.99 (d, $^3J = 8.4$ Hz, 1 H, 5-H), 4.97 (m, 1 H, 3′-H), 4.66 (m, 2 H, 2′-H, 4′-H), 3.7 (q, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.25 (s, 3 H, CH_3O), 3.1 (s, 3H, CH_3O), 3.18 (q, $^3J = 7.3$ Hz, 6 H, CH_2N), 1.9 (m, 8 H, CH_2CH_2O), 1.26 (t, $^3J = 7.3$ Hz, 9 H, CH_3CH_2N).

***R*$_P$ Isomer.** ^{1}H NMR (in D_2O): $\delta = 7.85$ (d, $^3J = 8.1$ Hz, 1 H, 6-H), 7.44−7.27 (m, 5 H, aromatic protons), 6.18 (d, $^3J = 7.7$ Hz, 1 H, 1′-H), 5.99 (d, $^3J = 8.1$ Hz, 1 H, 5-H), 4.96 (m, 1 H, 3′-H), 4.66 (m, 2 H, 2′-H, 4′-H), 3.7 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.23−3.15 (m, 12 H, CH_2N, CH_3O), 1.87 (m, 8 H, CH_2CH_2O), 1.25 (t, 9 H, CH_3CH_2N).

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(4-Chlorophenyl phosphorothioate) Triethylammonium Salt (2b). Yield: 44 mg (81%). ^{31}P NMR (in pyridine): $\delta = 52.4$ (0.62 P, S_P isomer) and 52.2 (0.38 P, R_P isomer). ^{1}H NMR (in D_2O): $\delta = 7.88$ (2 d, $^3J = 8.1$ Hz, 1 H, 6-H), 7.42 (d, $^3J = 8.8$ Hz, 2 H, aromatic protons), 7.26 (m, 2 H, aromatic protons), 6.24 (2 d, $^3J = 8.1$ Hz, 1 H, 1′-H), 6.04 (2 d, $^3J = 8.1$ Hz, 1 H, 5-H), 4.99 (dd, 1 H, 3′-H), 4.68−4.55 (m, 2 H, 2′-H, 4′-H), 3.75 (m, 8 H, CH_2O), 3.56 (m, 2 H, 5′-H), 3.28−3.11 (m, 12 H, CH_2-N, CH_3O), 1.9 (m, 8 H, CH_2CH_2O), 1.29 (t, 9 H, CH_3CH_2N). HRMS: found (M^-) 677.1361, $C_{27}H_{35}N_2O_{12}ClSP$ requires M, 677.1337.

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(3-Acetophenyl phosphorothioate) Triethylammonium Salt (2c). Yield: 46 mg (83%). ^{31}P NMR (in pyridine): $\delta = 52.4$ (0.70 P, S_P isomer) and 52.1 (0.30 P, R_P isomer). ^{1}H NMR (in D_2O): $\delta = 7.90-7.75$ (m, 3 H, 6-H, aromatic protons), 7.55 (d, 2 H, aromatic protons), 6.18 (2 d, 1 H, 1′-H), 6.02 (d, $^3J = 7.7$ Hz, 1 H, 5-H), 4.98 (m, 1 H, 3′-H), 4.67−4.55 (m, 2 H, 2′-H, 4′-H), 3.71 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.27−3.08 (m, 12 H, CH_2N, CH_3O), 2.64 (s, 3 H, CH_3CO), 1.86 (m, 8 H, CH_2CH_2O), 1.26 (t, 9 H, CH_3CH_2N). HRMS: found (M^-) 685.1878, $C_{29}H_{38}N_2O_{13}SP$ requires M, 685.1832.

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(3-Cyanophenyl phosphorothioate) Triethylammonium Salt (2d). Yield: 45 mg (83%). ^{31}P NMR (in pyridine): $\delta = 52.5$ (0.63 P, S_P isomer) and 52.1 (0.37 P, R_P isomer). ^{1}H NMR (in D_2O): $\delta = 7.85$ (2 d, $^3J = 8.4$ Hz, 1 H, 6-H), 7.69−7.55 (m, 4 H, aromatic protons), 6.18 (2 d, $^3J = 7.7$ Hz, 1 H, 1′-H), 6.02 (d, $^3J = 8.1$ Hz, 1 H, 5-H), 4.98 (dd, 1 H, 3′-H), 4.66−4.56 (m, 2 H, 2′-H, 4′-H), 3.71 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.28−3.07 (m, 12 H, CH_2N, CH_3O), 1.87 (m, 8 H, CH_2CH_2O), 1.26 (t, 9 H, CH_3CH_2N). HRMS: found (M^-) 668.1675, $C_{28}H_{35}N_3O_{12}SP$ requires M, 668.1679.

2′,5′-*O*-Bis(methoxytetrahydropyranyluridine) 3′-(2-Chlorophenyl phosphorothioate) Triethylammonium Salt (2e). Yield: 41 mg (75%). ^{31}P NMR (in pyridine): $\delta = 52.4$ and 52.2. ^{1}H NMR (in D_2O): $\delta = 7.91$ (2 d, $^3J = 8.1$ Hz, 1 H, 6-H), 7.51 (m 2 H, aromatic protons), 7.36 (t, 1 H, aromatic proton), 7.20 (t, 1 H, aromatic proton), 6.24 (2 d, $^3J = 8.1$ Hz, 1 H, 1′-H), 6.04 (d, $^3J = 8.1$ Hz, 1 H, 5-H), 5.06 (m, 1 H, 3′-H), 4.75−4.55 (m, 2 H, 2′-H, 4′-H), 3.75 (m, 8 H, CH_2O), 3.56 (m, 2 H, 5′-H), 3.28−3.11 (m, 12 H, CH_2N, CH_3O), 1.9 (m, 8 H, CH_2CH_2O), 1.29 (t, 9 H, CH_3CH_2N).

2′,5′-*O*-Bis(methoxytetrahydropyranyluridine) 3′-(3-Nitrophenyl phosphorothioate) Triethylammonium Salt (2f). Yield: 56 mg (80%). ^{31}P NMR (in pyridine): $\delta = 52.5$ (0.53 P, S_P isomer) and 52.1 (0.47 P, R_P isomer). ^{1}H NMR (in D_2O): $\delta = 7.92$ (m, $^3J = 8.1$ Hz, 2 H, 6-H, aromatic proton), 7.70 (m, 2H, aromatic protons), 7.39 (t, 1 H, aromatic proton), 6.21 (m, 1 H, 1′-H), 6.03 (d, $^3J = 8.1$ Hz, 1 H, 5-H),

4.99 (m, 1 H, 3′-H), 4.68−4.57 (m, 2 H, 2′-H, 4′-H), 3.77 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.28−3.07 (m, 12 H, CH_2N, CH_3O), 1.87 (m, 8 H, CH_2CH_2O), 1.28 (t, 9 H, CH_3CH_2N). HRMS: found (M^-) 688.1607, $C_{27}H_{35}N_3O_{14}SP$ requires M, 688.1577.

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(3, 5-Dichlorophenyl phosphorothioate) Triethylammonium Salt (2g). Yield: 25 mg (44%). ^{31}P NMR (in pyridine): $\delta = 52.4$ (0.57 P, S_P isomer) and 52.1 (0.43 P, R_P isomer). ^1H NMR (in D_2O): $\delta = 7.88$ (2 d, $^3J = 8.1$ Hz, 1 H, 6-H), 7.31 (m, 3 H, aromatic protons), 6.22 (2 d, $^3J = 7.7$ Hz, 1 H, 1′-H), 6.04 (2 d, $^3J = 8.1$ Hz, 1 H, 5-H), 4.98 (dd, 1 H, 3′-H), 4.66 (m, 2 H, 2′-H, 4′-H), 3.75 (m, 8 H, CH_2O), 3.56 (m, 2 H, 5′-H), 3.29−3.11 (m, 12 H, CH_2-N, CH_3O), 1.91 (m, 8 H, CH_2CH_2O), 1.29 (t, 9 H, CH_3CH_2N). HRMS: found (M^-) 711.0965, $C_{27}H_{34}N_2O_{12}Cl_2$-SP requires M, 711.0947.

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(4-Acetophenyl phosphorothioate) Triethylammonium Salt (2h). Yield: 50 mg (91%). ^{31}P NMR (in pyridine): $\delta = 51.8$ (0.64 P, S_P isomer) and 51.6.-(0.36 P, R_P isomer). ^1H NMR (in D_2O): $\delta = 8.03$ (d, $^3J = 8.8$, 2 H, aromatic protons), 7.83 (2 d, $^3J = 8.4$ and 8.1 Hz, 1 H, 6-H), 7.38 (m, $^3J = 8.8$, 2 H, aromatic protons), 6.16 (2 d, $^3J = 7.7$ and 11 Hz, 1 H, 1′-H), 5.99 (2 d, $^3J = 8.4$ Hz, 1 H, 5-H), 4.97 (m, 1 H, 3′-H), 4.68−4.57 (m, 2 H, 2′-H, 4′-H), 3.71 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.27−3.07 (m, 12 H, CH_2N, CH_3O), 2.6 (s, 3 H, CH_3CO), 1.86 (m, 8 H, CH_2CH_2O), 1.25 (t, 9 H, CH_3CH_2N). HRMS: found (M^-) 685.1839, $C_{29}H_{38}N_2O_{13}SP$ requires M, 685.1832.

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(4-Cyanophenyl phosphorothioate) Triethylammonium Salt (2i). Yield: 38 mg (70%). ^{31}P NMR (in pyridine): $\delta = 51.9$ (0.52 P, S_P isomer) and 51.6 (0.48 P, R_P isomer). ^1H NMR (in D_2O): $\delta = 7.84$ (d, $^3J = 8.4$ Hz, 1 H, 6-H), 7.79 (dd, $^3J = 8.4$ and 3.3 Hz, 2 H, aromatic protons), 7.42 (dd, $^3J = 7.0$ and 4.0 Hz, 2 H, aromatic protons), 6.16 (2 d, $^3J = 9.9$ Hz, 1 H, 1′-H), 6.0 (d, $^3J = 8.1$ Hz, 1 H, 5-H), 4.98 (dd, 1 H, 3′-H), 4.65−4.59 (m, 2 H, 2′-H, 4′-H), 3.71 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.28−3.07 (m, 12 H, CH_2-N, CH_3O), 1.87 (m, 8 H, CH_2CH_2O), 1.26 (t, 9 H, CH_3CH_2N). HRMS: found (M^-) 668.1678, $C_{28}H_{35}N_3O_{12}$-SP requires M, 668.1679.

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(2,5-Dichlorophenyl phosphorothioate) Triethylammonium Salt (2j). Yield: 33 mg (58%). ^{31}P NMR (in pyridine): $\delta = 52.5$ (0.56 P, S_P isomer) and 52.4 (0.44 P, R_P isomer). ^1H NMR (in D_2O): $\delta = 7.91$ (2 d, $^3J = 8.1$ Hz, 1 H, 6-H), 7.65 (d, $^3J = 1.5$ Hz, 1 H, aromatic proton), 7.51 (d, $^3J = 8.8$ 1 H, aromatic proton), 7.24 (d, $^3J = 8.4$, 1 H, aromatic proton) 6.24 (2 d, $^3J = 7.7$ Hz, 1 H, 1′-H), 6.03 (d, $^3J = 8.1$ Hz, 1 H, 5-H), 5.05 (dd, 1 H, 3′-H), 4.73−4.58 (m, 2 H, 2′-H, 4′-H), 3.79 (m, 8 H, CH_2O), 3.57 (m, 2 H, 5′-H), 3.29−3.13 (m, 12 H, CH_2N, CH_3O), 1.91 (m, 8 H, CH_2CH_2O), 1.29 (t, 9 H, CH_3CH_2N).

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(2-Nitrophenyl phosphorothioate) Triethylammonium Salt (2k). Yield: 27 mg (48%). RP HPLC ratio of diastereomers: 64% S_P isomer (retention time 56 min) to 36% R_P isomer (retention time 58 min) in 0.25 mM TEAHOAc buffer and 0−50% MeCN (100 min) as gradient. ^{31}P NMR (in pyridine): $\delta = 52.8$. ^1H NMR (in D_2O): $\delta = 8.15$ (dd, 2 H, aromatic protons), 7.85 (2 d, $^3J = 8.1$ Hz, 1 H, 6-H), 7.69 (m, 2 H, aromatic protons), 6.17 (m, 1 H, 1′-H), 6.0 (d, $^3J = 8.4$ Hz, 1 H, 5-H), 4.99 (m, 1 H, 3′-H), 4.68−4.57 (m, 2 H, 2′-H, 4′-H), 3.71 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H), 3.28−3.07 (m, 12 H, CH_2N, CH_3O), 1.87 (m, 8 H, CH_2CH_2O), 1.26 (t, 9 H, CH_3CH_2N).

2′,5′-*O*-Bis(methoxytetrahydropyranyl)uridine 3′-(4-Nitrophenyl phosphorothioate) Triethylammonium Salt (2l). Yield: 40 mg (72%). The ratio of diastereoisomers determined from integration of the reversed phase HPLC chromatogram was 52% S_P isomer to 48% R_P isomer. HRMS: found (M^-) 688.1625, $C_{27}H_{35}N_3O_{14}SP$ requires M, 688.1577.

***S*P Isomer.** ^1H NMR (in D_2O): $\delta = 8.32$ (d, $^3J = 8.9$ Hz, 2H, aromatic protons), 7.87 (d, $^3J = 8.4$ Hz, 1 H, 6-H), 7.47 (d, 2 H, aromatic protons), 6.20 (d, $^3J = 8.1$ Hz, 1 H, 1′-H), 6.02 (d, $^3J = 8.1$ Hz, 1 H, 5-H), 5.0 (m, 1 H, 3′-H), 4.64 (m, 2 H, 2′-H, 4′-H), 3.75 (m, 8 H, CH_2-O), 3.55 (m, 2H, 5′-H), 3.29−3.11 (m, 12 H, CH_3O, CH_2N), 1.9 (m, 8 H, CH_2CH_2O), 1.28 (t, 9 H, CH_3CH_2N).

***R*P Isomer.** ^1H NMR (in D_2O): $\delta = 8.32$ (d, $^3J = 8.9$ Hz, 2 H, aromatic protons), 7.88 (d, $^3J = 8.1$ Hz, 1 H, 6-H), 7.48 (d, 2 H, aromatic protons), 6.23 (d, $^3J = 7.7$ Hz, 1 H, 1′-H), 6.04 (d, $^3J = 8.1$ Hz, 1 H, 5-H), 5.0 (m, 1 H, 3′-H), 4.6 (m, 2 H, 2′-H, 4′-H), 3.75 (m, 8 H, CH_2O), 3.55 (m, 2 H, 5′-H),3.23−3.12 (m, 12 H, CH_3O, CH_2N), 1.87 (m, 8 H, CH_2CH_2O), 1.26 (t, 12 H, CH_3CH_2N).

Acknowledgment. We gratefully acknowledge financial support by the Swedish Natural Science Research Council and Procordia research funds.

JA953399D