Kinetics and Mechanisms for the Cleavage and Isomerization of the Phosphodiester Bonds of RNA by Brønsted Acids and Bases

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1. Introduction

Bioinorganic chemists' growing interest in the hydrolysis of RNA began in early 1980s, when it was discovered that not all biocatalysts are protein enzymes but that ribonucleic acids are also able to catalyze transesterification of their own phosphodiester bonds.[1,2] Very soon it was shown that the catalysis is not restricted to intrastrand transesterifications, but the action of the catalytic domains may also be targeted toward another RNA molecule.[3,4] Furthermore, the structure of these domains could be extensively modified without a marked loss of their catalytic activity.[5] It certainly was, and still is, a challenge for chemists to understand how the relatively stable phosphodiester linkages of RNA may be cleaved by another ribonucleotide sequence with an efficiency approaching that of protein enzymes, although the selection of reactive functionalities in RNA is rather modest. The longstanding endeavor to understand the action of protein ribonucleases at the level of individual bond making and breaking, and the application of this understanding to rational design of artificial enzymes, became accompanied by similarly motivated research on catalytic ribonucleic acids. Obviously the mechanistic studies on the cleavage of RNA by either ribonucleic acids or protein nucleases are both dependent on thorough understanding of the elementary processes involved in the nonenzymatic cleavage of RNA and the factors affecting the kinetics and thermodynamics of these processes. Accordingly, the interest in kinetics and mechanisms of the solvolytic reactions of RNA has rapidly expanded since late 1980s.

However, this is not the first time that the hydrolysis of RNA is the focus of interest. The early 1950s was a period of extensive and pioneering studies on RNA hydrolysis,[6,7] and the original observation on alkaline instability of RNA was much earlier.[8,9] In the 1950s the alkaline hydrolysis of RNA was mainly used as a tool to elucidate the structure of the internucleosidic linkage of ribonucleic acids. The first breakthrough took place early in the decade when it was established that each of the four different mononucleotides appeared in the alkaline hydrolyzate of RNA as a pair of isomers.[10−15] The isolated adenylic acids were observed to be identical with those obtained by phosphorylating 5′-*O*-trityladenosine,[16] they were readily interconverted in aqueous acid[13,15] but not in aqueous alkali,[16] and the alkaline hydrolysis of 2′,3′-cyclic nucleotide yielded a mixture of these two isomers much faster than the hydrolysis of RNA.[17] Accordingly, it seemed likely that 2′,3′-cyclic phosphates were the initial hydrolysis products of RNA, and they were subsequently hydrolyzed to a mixture of 2′- and 3′-monophosphates. Formation of nucleoside 2′,3′-cyclic phosphates could even be verified by a more controlled hydrolysis of RNA in aqueous barium carbonate and by digestion with ribonuclease.[18] RNA was later degraded to nucleoside 2′,3′-cyclic phosphates with *tert*-butoxide in formamide.[19] The question that remained was the assignment of the phosphodiester bonds either as 2′,5′- or 3′,5′-linkages. Since among the monomethyl and monobenzyl esters of isomeric nucleoside monophosphates, only the 3′-isomers were observed to be the substrates of pancreatic ribonuclease,[20] it became evident that the internucleosidic linkages are of the 3′,5′-type. This conclusion was widely accepted.[21−25] Accordingly, the facile alkaline cleavage of RNA could be accounted for by displacement of the 5′-linked nucleoside by deprotonated 2′-hydroxy group via a pentacoordinated transition state, the resulting 2′,3′-cyclic phosphate being rapidly hydrolyzed to a mixture of nucleoside 2′- and 3′-phosphates (Scheme 1).[26−28]

The early studies thus revealed the structure of RNA and explained the alkaline instability of RNA compared to that of DNA by the presence of an intramolecular nucleophile, the 2′-hydroxy function, in RNA. These explanations still hold. After this period of rapid progress, the chemical hydrolysis of RNA or its fragments was investigated as part of

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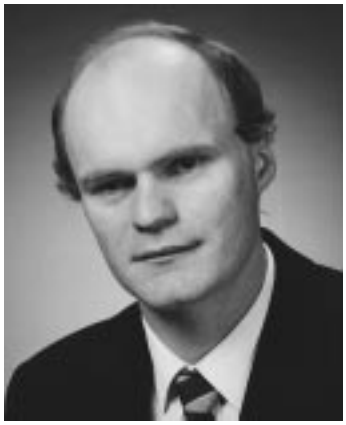

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mechanistic studies of the action of ribonucleases. However, quantitative kinetic data for the reactions of real nucleotides remained scanty, largely due to the lack of appropriate methods with which to follow accurately the relatively slow reactions that often led to complicated product mixtures. Meanwhile, the recognition of the central role of phosphates in the living world gave rise to extensive kinetic and mechanistic studies on solvolytic reactions of more simple phosphoric acid esters.[29,30] The emphasis of these investigations was on the factors that govern the formation, isomerization, and breakdown of the pentacoordinated intermediate obtained by the attack of a nucleophile on tetracoordinated pentavalent phosphorus. Westheimer summarized the experimental observations in 1968 by presenting his well-known concept on pseudorotating pentacoordinated oxyphosphorane intermediates.[31] Since then this concept has been used as the guidelines for mechanistic discussions concerning the hydrolysis of phosphate esters. It may well be regarded as the second milestone in the history of RNA hydrolysis. According to this theory, the attack of nucleophile on a tetrahedral phosphate ester gives a pentacoordinated

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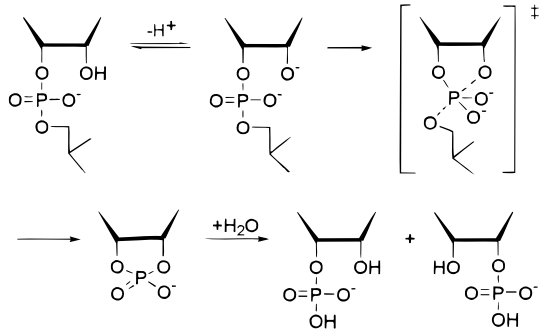
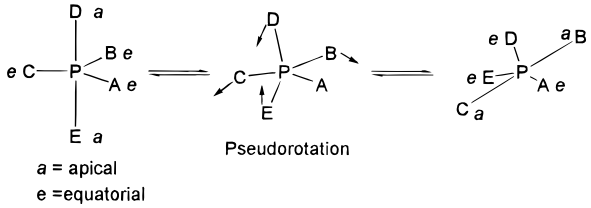
Scheme 1



Scheme 2



species, the structure of which is a trigonal bipyramid having two apical and three equatorial ligands (Scheme 2). The P−O bond of an apical ligand is longer than that of an equatorial ligand. Nucleophiles may enter and leave the intermediate only through apical positions, but the pentacoordinated phosphorane may be sufficiently stable to allow ligand reorganization by a pseudorotation process: one of the equatorial ligands remains equatorial, while the other two take an apical position and the originally apical ligands become equatorial. The chemical nature of the ligands affects their apicophilicity; electronegative ligands prefer an apical position and electropositive an equatorial position. As far as transesterification reactions of RNA are concerned, it is important to note that the attack of the neighboring sugar hydroxy group on phosphorus results in the formation of a five-membered ring.

Initially the attacking oxygen must be apical. According to Westheimer's concept, the other oxygen atom of this five-memberd ring must then be equatorial: diapical and diequatorial five-membered rings were assumed to be high-energy structures. Recent ab initio calculations have confirmed this assumption.[32] Accordingly, transesterification of a 3′,5′-diester to the 2′,5′-isomer may only occur via an oxyphosphorane intermediate which is sufficiently stable to pseudorotate. The impact of Westheimer's concept on the mechanistic chemistry of RNA phosphodiester bonds cannot be overestimated. The discussions of the present review are also entirely based on these guidelines.

The discovery of catalytic ribonucleic acids constitutes the third milestone of RNA hydrolysis. As mentioned in the beginning of this introduction, the interest in mechanistic chemistry of RNA has rapidly expanded during the past decade largely due to the desire to understand the action of catalytic ribonucleic acids. More concrete reasons may also be found. On the one hand, developments in synthetic chemistry of nucleic acid constituents have enabled preparation of structurally complicated model compounds in sufficient amounts and modern, sensitive, analytical techniques, such as HPLC, capillary electrophoresis, and NMR spectroscopy, allow quantitative monitoring of complicated reaction systems, consisting of several parallel and consecutive partial reactions. On the other hand, the studies on solvolytic reactions of simple phosphate esters have provided the necessary mechanistic background, and advanced methods of quantum chemical calculations may now be used to evaluate critically kinetically equivalent mechanisms. Finally, the rapid progress in molecular biology, especially in selective inhibition of gene expression by structurally modified oligonucleotides,[33−36] has created a need for artificial catalysts that could sequence selectively cleave RNA.[37,38] The rational design of such agents must be based on thorough understanding of the catalysis mechanisms involved.

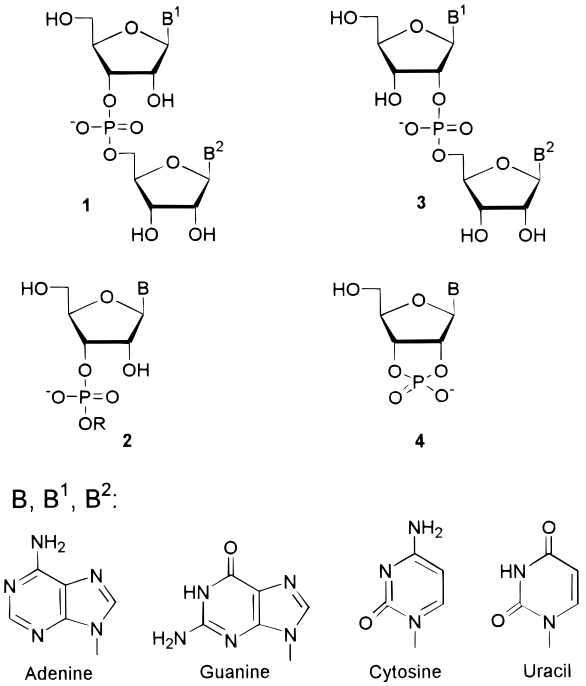
This review summarizes the existing quantitative data on cleavage and isomerization of RNA phosphodiester bonds by Brønsted acids and bases. Accordingly, the extensive literature dealing with the role of metal ions in RNA hydrolysis is outside the scope of this review. The results of quantum chemical calculations are also referred only when they help to distinguish between alternative mechanisms derived from experimental observations. The emphasis is on studies carried out with compounds that closely mimic the structure of RNA constituents, but supporting evidence based on more modified structures is also frequently discussed.

2. Specific Acid/Base Catalyzed Cleavage and Isomerization of the 3′,5′-Phosphodiester Bonds

A. Minimal Reaction Scheme

As discussed in the Introduction and depicted in Scheme 1, the early studies on RNA hydrolysis showed that the RNA phosphodiester bonds are cleaved in aqueous alkali by intramolecular displacement of the 5′-linked nucleoside by the 2′-oxyanion,

Chart 1

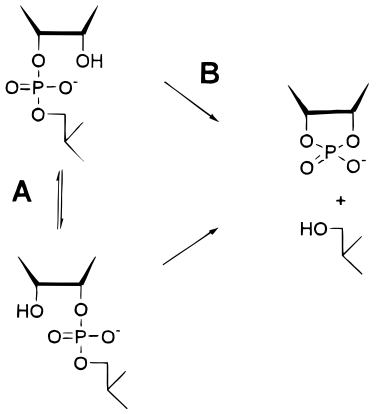


giving 2′,3′-cyclic phosphates that are rapidly hydrolyzed to a mixture of 2′- and 3′-phosphates. Under less basic conditions, the 3′,5′-bond, however, undergoes an additional intramolecular transesterification: reversible isomerization to a 2′,5′-bond (reaction **A** in Scheme 3) takes place along with the cyclization to a 2′,3′-cyclic phosphate (reaction **B** in Scheme 3). The occurrence of these reactions has been established with dimeric fragments of RNA, i.e., dinucleoside 3′,5′-phosphates (3′,5′-NpN; **1**, Chart 1),[39,40] ribonucleoside 3′-(alkyl phosphates) (**2**, Chart 1),[41] and polyribonucleotides.[42] Owing to rapid acid-catalyzed hydrolysis of 2′,3′-cyclic phosphates to 2′- and 3′-phosphates,[43−47] the cyclic phosphates can be detected as intermediates only under alkaline conditions. However, in acidic solutions, 3′,5′-NpN (**1**) yields nucleoside 2′- and 3′-phosphates in exactly the same ratio ([2′]/[3′] = 0.6) as the much faster hydrolysis of 2′,3′-cyclic phosphates,[40,41] although the mutual isomerization of the 2′- and 3′-phosphates is too slow to result in equilibration under the reaction conditions.[46,48] When the 5′-linked nucleoside is replaced with a better leaving group, such as 2-chlorophenoxy group, the intermediary appearance of the 2′,3′-cyclic phosphate is clearly observed even at pH 2.[49] Accordingly, there seems to be no reason to believe that the course of the reactions depicted in Scheme 3 would somehow be changed on going to acidic solutions. In reality, the situation is more complicated than indicated in Scheme 3, since the reactions taking place at the base moieties or the anomeric carbon may compete with the transesterifications. Furthermore, nucleoside 2′- and 3′-phosphates are subsequently hydrolyzed to nucleosides. These competing and subsequent reactions are briefly discussed in later sections of this review.

B. The Reactive Ionic Forms

The pH−rate profiles for both of the transesterifications depicted in Scheme 3 have been determined

Scheme 3



over a wide range of acidity, extending from concentrated acid solutions to concentrated aqueous alkalies. Figures 1a and 2a show the buffer-independent rate constants obtained with uridylyl(3′-5′)uridine (3′,5′-UpU; **1**, $B^1 = B^2 =$ uracil).[40] As seen, the cleavage (reaction **B**) is catalyzed by both hydronium and hydroxide ions, while the isomerization (reaction **A**) is susceptible only to acid catalysis. The shape of the pH−rate profiles has been interpreted to indicate involvement of four kinetically distinct terms in the cleavage reaction, and three terms in the isomerization, viz. dependence of rate on $[SH_2][H^+]$, $[SH_2]$, $[SH^-]$, and $[S^{2-}]$ with the cleavage, and on $[SH_2][H^+]$, $[SH_2]$, and $[SH^-]$ with the isomerization.[40] Here SH_2 stands for neutral phosphodiester, SH^- monoanionic phosphodiester, and S^{2-} monoanionic diester having additionally the 2′-hydroxy deprotonated (Figure 3). Figures 1b and 2b show the percent contribution of the various kinetic terms to the observed rate of cleavage and isomerization as a function of pH (calculated on the basis of the data reported in ref 40).

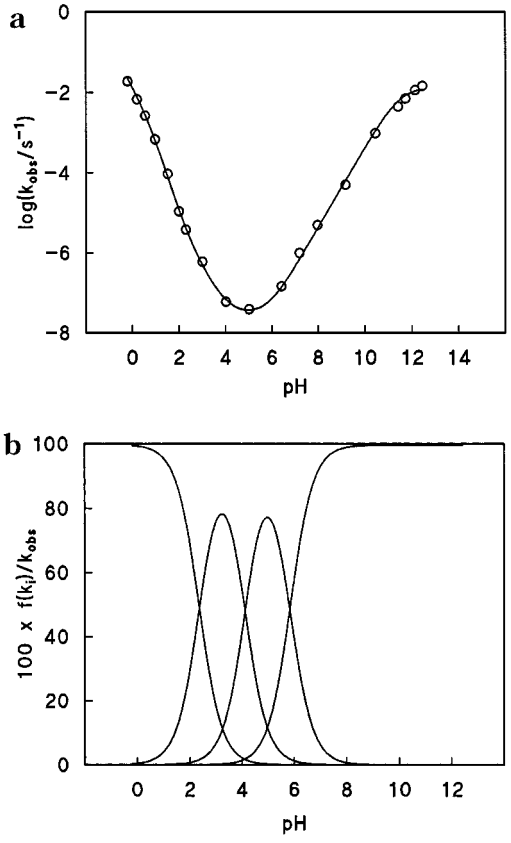


Figure 1. pH−rate profile for the cleavage of 3′,5′-UpU at 90 °C (a),[40] and the percent contribution of various kinetic terms to the observed rate constant (b, the partial reactions from left to right refer to the dependence of rate on $[SH_2][H^+]$, $[SH^2]$, $[SH^-]$, and $[S^{2-}]$). For SH_2, SH^-, and S^{2-} see Figure 3.

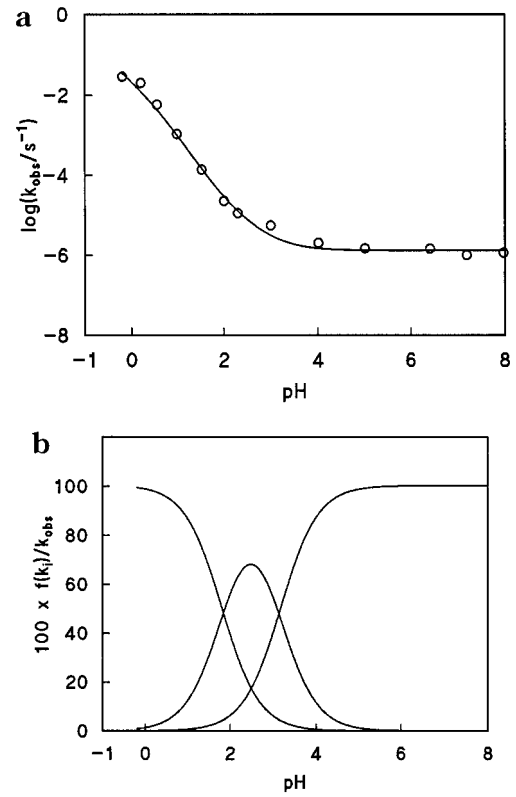


Figure 2. pH−rate profile for the mutual isomerization of 3′,5′-UpU and 2′,5′-UpU at 90 °C (a),[40] and the percent contribution of various kinetic terms to the observed rate constant (b, the partial reactions from left to right refer to the dependence of rate on $[SH_2][H^+]$, $[SH^2]$, and $[SH^-]$). For SH_2, SH^-, and S^{2-} see Figure 3.

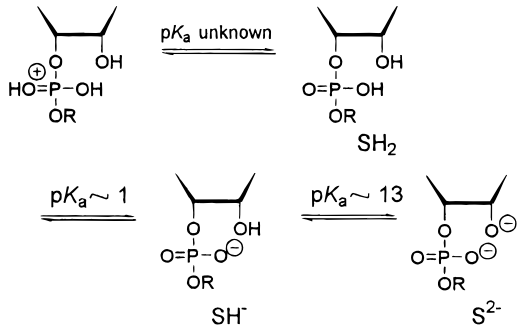
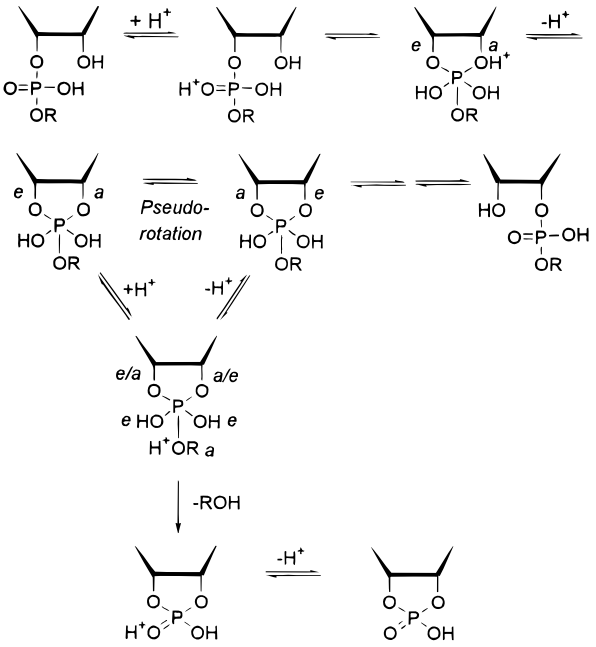


Figure 3. Protolytic equilibria of an internucleosidic 3′,5′-phosphodiester bond.

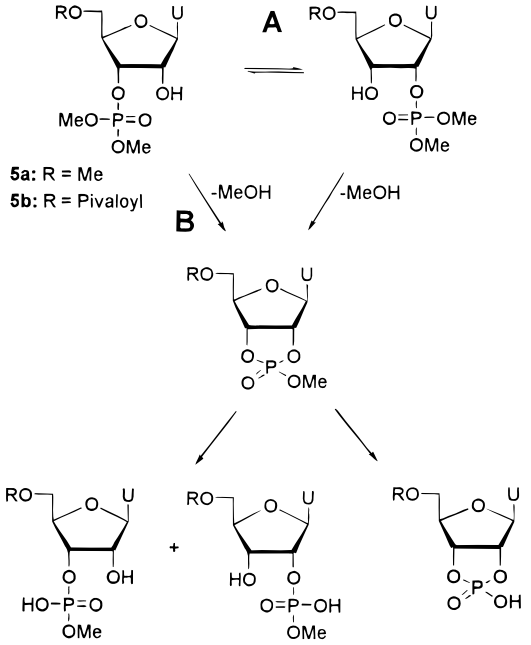
C. Hydronium Ion Catalyzed Reactions of Neutral Phosphodiester

The term referring to the dependence of rate on $[SH_2][H^+]$ predominates at pH < 2 with both the cleavage and isomerization. This term has been

Scheme 4



Scheme 5

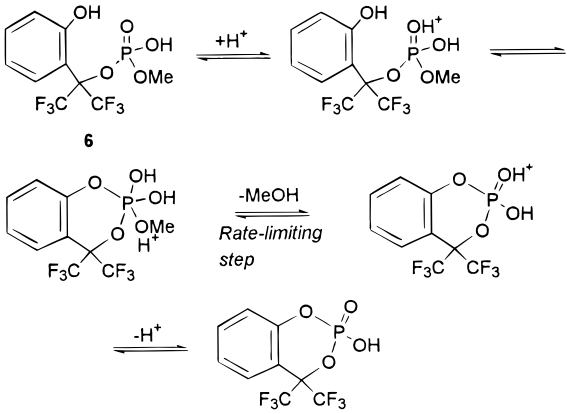


attributed to rapid initial protonation of the nonbridging phosphoryl oxygen, followed by attack of the 2′-hydroxy group on phosphorus (Scheme 4).[40,41] Accordingly, a monocationic phosphorane intermediate having the attacking 2′O in apical and the 3′O in equatorial position is initially obtained. This intermediate may collapse back to the starting material, but it may also undergo rapid deprotonation to a neutral form, which is the stable ionic form of the phosphorane intermediate under these conditions. The estimates for the first pK_a value of oxyphosphoranes range from 6.5 to 11.[50−52] According to ab initio calculations, a neutral phosphorane represents a clear potential energy minimum in gas phase, and is sufficiently stable to pseudorotate.[53] Pseudorotation may hence transfer the 3′O to an apical position, and subsequent protonation of the 3′O and departure of the 3′-OH then completes the isomerization. Alternatively, the 5′O may take an apical position, either initially or after pseudorotation, and hence the 5′-linked nucleoside may depart, resulting in the cleavage reaction. The isomerization of 3′,5′-UpU to 2′,5′-UpU (**3**, $B^1 = B^2$ = uracil; Chart 1) and its reverse reaction are approximately as fast, and slightly slower than their cyclization to the 2′,3′-cyclic phosphate (**4**, B = uracil), the relative rates being 1, 0.9, and 1.2, respectively.[40] These relative rates are independent of pH, as long as the reaction referring to dependence of rate on $[SH_2][H^+]$ is concerned. Therefore the pseudorotation can hardly be the rate-limiting step of isomerization. As indicated in Scheme 4, a neutral phosphorane having 2′O apical and 3′O equatorial may undergo three reactions: (i) protonation of 2′O and concomitant collapse to the starting material, (ii) pseudorotation leading to isomerization, and (iii) protonation of 5′O leading to cleavage. Obviously reactions i and iii are hydronium ion catalyzed, while ii is an uncatalyzed process. In case of uncatalyzed rate-limiting pseudorotation, the cleavage should become favored over isomerization on increasing the hydronium ion concentration. Since this is not the case, it appears reasonable to assume that the phosphoranes having either the 2′O or 3′O apical are rapidly equilibrated via pseudorotation.

Isomerization and cleavage of nucleoside 3′-(dimethyl phosphates) (**5a**,**b**; reactions **A** and **B** in Scheme 5) have been studied as a model of the corresponding reactions of the neutral ionic form (SH_2) of nucleoside 3′-(methyl phosphate) (**2**; R = Me).[54,55] The results are consistent with the mechanism described above and suggest **5a**,**b** to be a reasonably good mimetic for a neutral diester. While the cleavage and isomerization of **2** (R = Me) exhibits a gradual change from second-order to first-order dependence of rate on hydronium ion concentration on passing the p$K_a \approx 1$ of the phosphodiester group,[41] the corresponding reactions of **5a**,**b** are strictly first-order in hydronium ion concentration over this acidity range.[54,55] In other words, the additional methyl group in the triester has clearly taken the role of the phosphoryl hydroxy proton of **2**, and both reactions proceed via a monocation. In fact, even the absolute reaction rates observed with **5b** and **2** are comparable.[41,55] Both compounds behave similarly also in the sense that the cleavage is about 3 times as rapid as the isomerization.

An analogous stepwise mechanism has been suggested for the hydronium ion catalyzed cyclization of phosphodiester **6** (Scheme 6).[56] The cyclization is also catalyzed by general acids, but exhibits only low susceptibility to the pK_a value of the buffer acid, consistent with the assumed breakdown of a high-energy phosphorane intermediate. No phosphate migration takes place, which has been attributed to thermodynamically unfavorable pseudorotation: the high apicophilicity of the phenoxy group is reinforced by steric preference of the bulky hexafluoroisopropoxy group for the equatorial position.

Scheme 6



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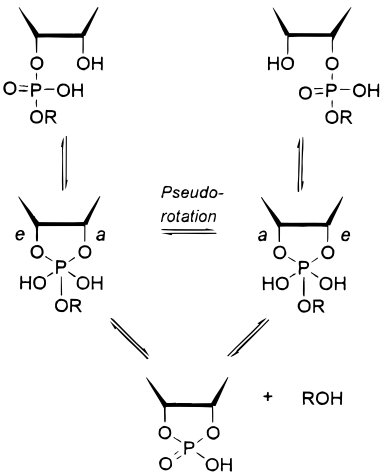
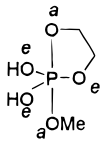
D. Uncatalyzed Reactions of Neutral Phosphodiester

The kinetic term referring to the dependence of rate on [SH_2] predominates both with the isomerization and cleavage over a relatively narrow acidity range around pH 3. As with the hydronium ion catalyzed reaction of SH_2, the isomerization and cleavage take place approximately as readily, the relative rates for the isomerization of 3′,5′-UpU to 2′,5′-UpU, its reverse reaction and cyclization to the 2′,3′-cyclic phosphate being 1, 0.8, and 0.3, respectively.[40] The most straightforward mechanistic explanation is the one depicted in Scheme 7. The attack of 2′-hydroxy function on neutral phosphate is accompanied by water-mediated proton transfer from the attacking nucleophile to the phosphoryl oxygen, the neutral phosphorane obtained rapidly pseudorotates, and either P−O3′ or P−O5′ bond is cleaved concerted with water-mediated proton transfer from the phosphoryl hydroxy function to the leaving oxygen. This mechanism is consistent with the results of ab initio calculations on the neutral phosphorane **7** derived from methyl ethylene phosphate.[53] According to these calculations, pseudorotation is faster than either endocyclic or exocyclic PO bond rupture, barriers for these processes being 30 (pseudorotation), 120 (endocyclic cleavage), and 150 kJ mol^{-1} (exocyclic cleavage). Both the endocyclic and exocyclic cleavages have been suggested to be

Scheme 7





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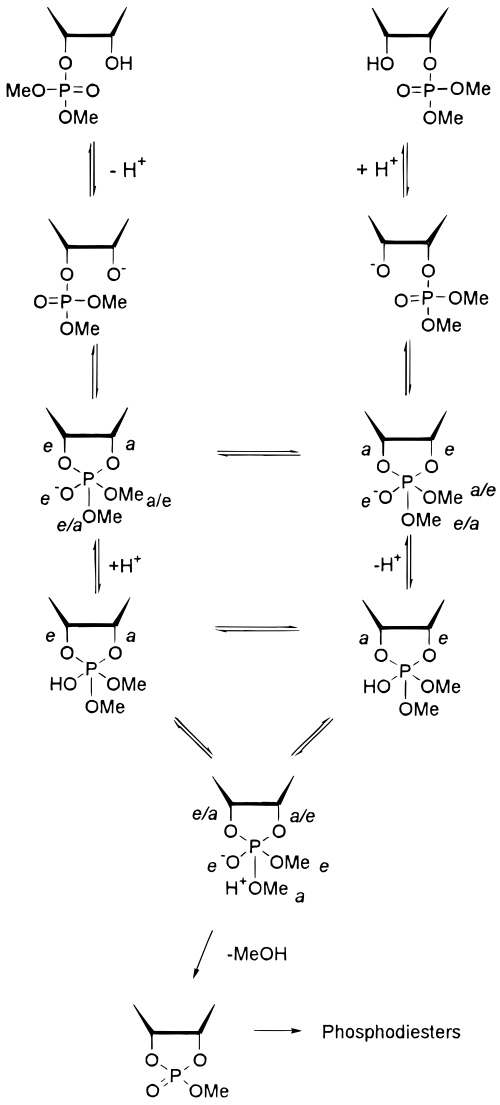
accompanied by an intramolecular proton transfer from the phosporyl hydroxy ligand to the departing oxygen. An alternative explanation for the observed dependence of rate on [SH_2] is the attack of 2′-oxyanion on monocationic phosphate. The reverse reaction would then be rupture of the P−O bond without concomitant proton transfer from the phosphate hydroxy ligand to the departing alkoxide ion. This appears to be a more difficult process than the one involving such a proton transfer. In as far as no experimental evidence for this kind of mechanism exists, the mechanism via the major tautomer must be preferred.

E. Uncatalyzed Reactions of Phosphodiester Monoanion

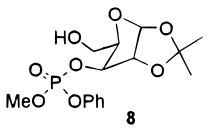
The isomerization is pH-independent in the pH range 4−8 (Figure 2a,b).[40,41] In all likelihood this is the case even at higher pH, but owing to rapid hydroxide ion catalyzed cleavage (Figures 1a and 2a), isomerization cannot be detected under such conditions. A pH-independent cleavage has also been suggested to exist, but only over a narrow range around pH 5 (Figure 1b).[40] In striking contrast to the situation under more acidic conditions, the pH-independent isomerization is much faster than cyclization, the relative rates for the isomerization of 3′,5′-UpU to 2′,5′-UpU, its reverse reaction, and cleavage to 2′,3′-cyclic phosphate being 1, 1, and 0.04, respectively.[40] In fact, the rate constant of pH-independent cleavage is based on only one experimental point, which shows a small positive deviation from the linear parts of the rate profile representing the dependence of rate on [H^+] and [H^+]$^{-1}$, respectively. That is why the pH-independent reaction may actually be even much slower than reported. Anyway, it is clear that the isomerization prevails over cyclization at pH 3 to 6, while the hydroxide ion catalyzed cleavage becomes the dominating reaction at pH > 6.[40]

The mechanisms of the pH-independent reactions are open to various interpretations, and of considerable interest since they represent the situation under biologically relevant neutral conditions. As discussed originally by Usher et al.,[57] the dependence of rate on [SH^-] may be accounted for by two kinetically equivalent mechanisms: the attack of the 2′-hydroxy group on the phosphodiester monoanion, i.e., the reaction via the predominant ionic form, or the attack of 2′-oxyanion on neutral phosphodiester. Although the latter ionic form is a minor tautomer, the attack of alkoxide ion on neutral phosphate may be expected to be so much more facile than the attack of alcohol on phosphate monoanion that this mechanism cannot apriori be rejected. Furthermore, the catalysis of the breakdown of the phosphorane intermediate may also contribute to the formal kinetics followed.

Scheme 8



The results obtained with nucleoside 3′-(dimethyl phosphate) (**5b**) have been used to distinguish between the kinetically equivalent mechanisms.[55] As indicated above, **5b** appears to be a reasonable model of a neutral diester. The isomerization of **5b** to its 2′-counterpart (Scheme 5) becomes hydroxide ion catalyzed already at pH > 2, suggesting that 2′-oxyanion is the attacking nucleophile (Scheme 8).[55] This may appear somewhat unexpected, since it means that the 2′-oxyanion should be approximately 10^{10} times as good a nucleophile as the 2′-hydroxy function (the pK_a value of the 2′-OH may be estimated to be 13 under the reaction conditions[40]). The studies on diester **6** and triester **8**, however, seem to be consistent with this interpretation. First, the



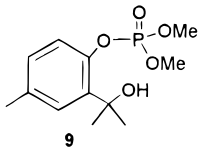
8

cyclization of the monoanion of **6** has also been deduced to proceed via the minor tautomer, i.e., by the attack of phenoxide ion on neutral phosphodiester, although the mole fraction of this species is only 2×10^{-9}.[56] Second, the cyclization of **8** by displacement of the phenoxy group by the hydroxymethyl function is hydroxide ion catalyzed at pH as low as 5.[58] Unfortunately no experimental data is available at lower pH. Bearing in mind that the 5′-hydroxy group is less acidic than the 2′-function, this means that again the oxyanion must be up to 10^{10} times as efficient a nucleophile as the hydroxy group.

An attack of un-ionized 2′-hydroxy group on phosphorus and subsequent deprotonation of the neutral phosphorane obtained might be envisaged as an alternative explanation for the inverse dependence of the isomerization rate of **5b** on acidity. If it is assumed that the monoanionic phosphorane undergoes rate-limiting pseudorotation much more rapidly than the neutral phosphorane, a first-order dependence of rate on hydroxide ion concentration is observed at pH < the first pK_a value of oxyphosphorane. Recent ab initio calculations do not, however, lend support for this alternative. The barrier heights calculated for the pseudorotation of a neutral and monoanionic **7** did not differ markedly.[53] We also know that pseudorotation of the neutral phosphorane must be relatively facile, since the isomerization of **5b** efficiently competes with cleavage under very acidic conditions.[40,41] Accordingly, the isomerization of **5b** at pH > 2 in all likelihood involves a rapid initial deprotonation of the 2′-hydroxy function, attack of the resulting oxyanion on phosphorus, and uncatalyzed pseudorotation of the monoanionic phosphorane, which may also take place via intermediary kinetically invisible protonation to neutral phosphorane (Scheme 8).

While the isomerization of **5b** becomes hydroxide ion catalyzed at pH > 2, the cleavage remains pH-independent up to pH 7, and shows hydroxide ion catalysis only at pH > 7.[55] If it is accepted that both reactions take place via the same intermediate, which appears logical, the breakdown of the intermediate to the cleavage products must be acid catalyzed. In other words, while the 2′- and 3′-oxygen ligands may leave as oxyanions, the methoxy ligand does so 10^5 times less readily, and hence it undergoes acid-catalyzed departure at pH < 7. The fact that the sugar hydroxy functions are 2 to 3 orders of magnitude more acidic than methanol offers only a partial explanation for the dissimilar behavior. Undoubtedly the preferred endocyclic fission also reflects some intrinsic property of the cyclic oxyphosphorane structure. Quantum chemical calculations on both mono- and dianionic phosphoranes suggest that the endocyclic PO bond is weaker than the exocyclic one.[57,59−68] The ring strain in the phosphorane intermediate weakens the apical endocyclic P−O bond compared to the exocyclic one. Finally, it is worth noting that cyclization of phosphotriester **9** shows a pH−rate profile similar to that of the cleavage of **5b**: the reaction is pH-independent up to pH 8, and turns then base-catalyzed.[69]

The preceding discussion helps to distinguish between the kinetically equivalent mechanisms of the mutual isomerization of 2′,5′- and 3′,5′-NpN. The 2′-oxyanion appears to be up to 10^{10} times better nucleophile than the 2′-hydroxy group. Moreover, it



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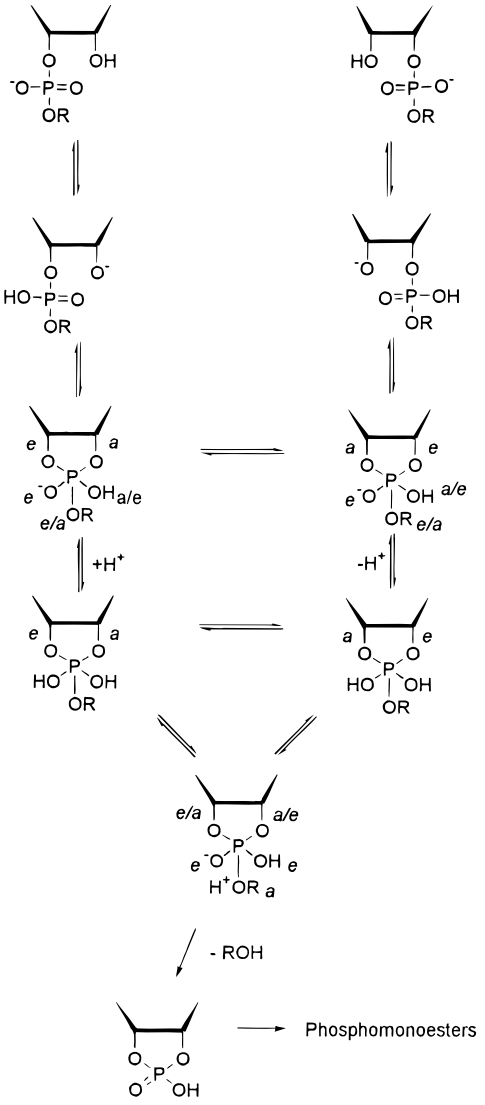
has been shown that a neutral phosphate group is attacked by a neighboring oxyanion up to 10^5 times as fast as a monoanionic one.[58,70] These two facts together have added weight to the conclusion that the pH-independent isomerization of 2′,5′- and 3′,5′-phosphodiester bonds proceeds by an attack of the neighboring oxyanion on neutral phosphodiester bond, followed by pseudorotation of the monoanionic phosphorane, either as a monoanion or, at lower pH, via kinetically invisible protonation to a neutral phosphorane (Scheme 9). The breakdown of the

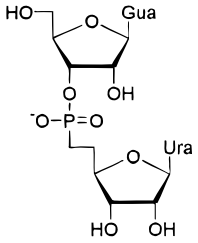
Scheme 9



intermediate to 2′,3′-cyclic phosphate with release of 5′-linked nucleoside as alkoxide ion may be expected to be much slower, as described above for **5b**. It is difficult to decide whether the pseudorotation limits the rate of isomerization. The pseudorotation barrier for the monoanionic **7** has been reported to be of the order of 30 kJ mol^{-1},[57] while a value of 20 kJ mol^{-1} has been given for the endocyclic PO bond cleavage of the same species.[62] Accordingly, the possibility that pseudorotation is at least partially rate-limiting cannot be excluded.

F. Cleavage via a Dianionic Phosphorane Intermediate/Transition State

The cleavage of 3′,5′-UpU becomes hydroxide ion catalyzed at pH > 6.[40] The reaction shows strict first-order dependence on hydroxide ion concentration up to pH 12, and then the rate levels off to a constants value under conditions where the 2′-hydroxyl function may be expected to undergo deprotonation (Figure 1a). Accordingly, a rapid initial deprotonation of the 2′-hydroxy group and subsequent attack of the oxyanion on phosphodiester monoanion appears to take place, as previously suggested in 1950s (Scheme 1). By contrast, hydroxide ion catalyzed isomerization has never been reported. One might speculate that even if a hydroxide ion catalyzed isomerization existed, it might be difficult to be detected, owing to concurrent rapid cleavage. Studies on the phosphonate analogue **10** of 3′,5′-GpU argue, however, against this explanation.[71] Compound **10**

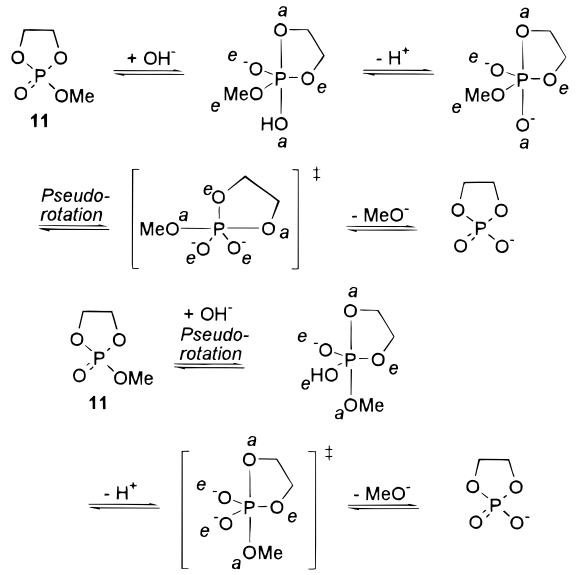


10

undergoes pH-independent isomerization even slightly faster than 3′,5′-UpU, which shows that the monoanionic and/or neutral pentacoordinated intermediate derived from **10** pseudorotates as easily as the one derived from 3′,5′-UpU, although both $-O^-$ and $-CH_2R$ as electropositive ligands may be expected to prefer equatorial position. Evidently rapid prototopic tautomerism between the nonbridging oxygens enable facile pseudorotation. The isomerization of **10** remains pH-independent up to pH 11. At higher pH, degradation of the uracil base[72] prevented the measurements. Accordingly, the dianionic pentacoordinated species, which undoubtedly is formed with **10** at such high pH, seems to be too unstable to pseudorotate, or actually to be stabilized by protonation to a monoanionic phosphorane known to pseudorotate. Since the second pK_a value of a phosphorane intermediate is believed to range from 11 to 15,[50−52] such a protonation should, under mildly alkaline conditions, be thermodynamically favored, and hence almost a diffusion controlled process. The lack of hydroxide ion catalyzed isomerization must hence be taken as an indication of extreme instability of the dianionic phosphorane intermediate; when formed it rapidly collapses back to the starting material.

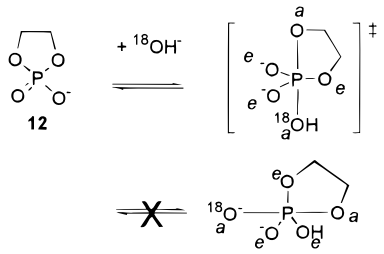
Perreault and Anslyn[52] have recently reviewed the experimental evidence in favor or against the existence of a dianionic phosphorane intermediate. Their

Scheme 10



conclusion is that such an intermediate, if it exists, cannot be distinguished kinetically from a transition state. According to ab initio calculations, a cyclic dianionic phosphorane does not show a potential energy minimum in gas phase,[67,73,74] or only a very shallow one,[61,63,75,76] but it has been repeatedly suggested that solvation may stabilize the structure to such an extent that the dianionic phosphorane ought to be considered as an intermediate, not only as a transition state.[66,77] Another experimental observation that one might conclude is in support of the intermediate nature of dianionic phosphorane is that methyl ethylene phosphate (**11**) has been shown to undergo in concentrated aqueous alkali exocyclic fission to a small but measurable extent by a mechanism that requires second-order dependence of rate on acidity.[78,79] Several lines of evidence indicate that the second-order dependence does not result from formation of a hexacoordinated intermediate.[80−83] Consequently, it has been argued that the originally apical hydroxy ligand is deprotonated, and this forces the resulting oxyanion to equatorial position (Scheme 10a).[83] In other words, the dianionic phosphorane would pseudorotate. The quantum chemical studies of Lim and Tole[62] offer, however, an alternative explanation. Pseudorotation takes place in concert with the attack of hydroxide ion. Accordingly, the methoxy ligand is apically bonded already in the initially obtained monoanionic phosphorane, and deprotonation of the equatorial hydroxy ligand may hence lead to exocyclic cleavage without pseudorotation (Scheme 10b). Consistent with the instability of dianionic phosphorane, ^{18}O is not incorporated into ethylene phosphate (**12**) under alkaline conditions.[84] An attack of isotopically labeled hydroxide ion on the phosphodiester monoanion gives a dianionic phosphorane having the labeled hydroxy ligand apical and the unlabeled oxyanions equatorial (Scheme 11). Since the isotopic label is not incorporated into the starting material, the dianionic phosphorane seems to be too short-lived to undergo the protolytic rearrangement and pseudorotation needed to bring the isotopically labeled oxygen in equatorial position. On

Scheme 11



the basis of these observations and the lack of hydroxide ion catalyzed isomerization of **10**, the conclusion of Perreault and Anslyn[52] that a transition state like dianionic phosphorane may exist but cannot be stabilized by protonation to a monoanionic phosphorane appears reasonable.

3. General Acid/Base Catalyzed Cleavage and Isomerization of the 3′,5′- Phosphodiester Bonds

A. General

The cleavage and isomerization of the 3′,5′-phosphodiester bonds are also susceptible to catalysis by general acids and bases. These reactions have actually attracted more attention than their specific acid/base catalyzed counterparts. The results of these studies have been recently reviewed.[52,85]

Most of the studies have been carried out in imidazole buffers to elucidate the possible catalysis mechanisms of RNase A, in which the key catalytic groups are the imidazole groups of two histidine residues.[86] The buffer catalysis is, however, rather modest, and concentrated buffer solutions have had to be used in kinetic measurements. 3′,5′-UpU, for example, has been shown to be cleaved in 1 mol L^{-1} imidazole buffer ($[Im]/[ImH^+] = 1$) only three times as fast as at buffer concentration zero.[39] The catalysis is even less pronounced with the isomerization: in the same buffer only a 25% rate acceleration was observed. Accordingly, the cleavage is usually faster than isomerization in buffers,[39,85] although the opposite is true with the buffer-independent reactions at pH < 7.3.[40] The inefficiency of the general catalysis has caused experimental problems. Complete elimination of medium effects of the buffer constituents is difficult at high buffer concentrations, and the risk of misinterpretation of experimental results is obvious even when care is exercised. Finally, one should note that the mechanism of buffer catalysis may depend on the basicity of the buffer system. The cleavage is first-order in the total concentration of imidazole or morpholine buffers, as shown with 3′,5′-UpU[39,87] and 3′,5′-ApA (**1**; $B^1 = B^2$ = adenine),[88] and polyuridylic acid [poly(U)].[89] By contrast, a second-order dependence of rate on buffer concentration is observed with more acidic acetic acid buffers.[39,85] Since rather extensive studies have been carried out in imidazole and morpholine buffers, and the action of these buffer may possibly mimic some features of the enzyme catalysis, the conclusions drawn from these investigations are first considered.

B. Catalysis by Imidazole and Morpholine Buffers

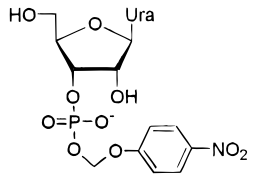
The cleavage of the 3′,5′-phosphodiester bond is first-order in the total concentration of imidazole and

morpholine buffers. It has been repeatedly suggested that both the acidic and basic buffer constituents serve as a catalyst.[39,85,87−93] The most convincing evidence of this was obtained when the cleavage of 3′,5′-UpU was studied in morpholine buffers.[87] The slope of the plot of the observed rate constant against buffer concentration clearly increased on increasing the buffer ratio [B]/[BH^+]. This as such could well be accounted for by mere general base catalysis. Additional catalysis by the buffer acid was, however, established by an experiment where the concentration of buffer base was kept constant and that of buffer acid was increased. The addition of the acidic buffer constituent clearly resulted in a rate increase,[85] even though the concomitant decrease in pH retards the buffer-independent cleavage.[40]

The participation of both buffer constituents in the catalysis had earlier been suggested on the basis of bell-shaped pH−rate profiles observed for the cleavage of 3′,5′-UpU and 3′,5′-ApA at a constant total concentration of imidazole buffer.[39,88,89] These measurements had, however, been carried out without proper control of the ionic strentgh. When the ionic strength was adjusted to 1.0 mol L^{-1}, the bell-shaped pH−rate profile disappeared, and the dependence of rate on the protonation state of the buffer ([BH^+]/[$B]_{tot}$) became approximately linear.[85] In other words, although the original observation on a bell-shaped pH−rate profile does not seem to hold, the conclusion drawn on the basis of this observation may still be valid. In fact, the shape of the buffer-dependent pH−rate profile seems to be rather sensitive to the ionic strength. The second-order rate constants for the imidazole catalysis has been observed to level off with increasing pH at the ionic strength of 0.5 mol L^{-1}, but not at the ionic strength of 1.0 mol L^{-1}.[94] Anyway, disappearance of the bell-shaped pH−rate profile needs an explanation. If both the buffer acid and buffer base are required for efficient catalysis and still the reaction is first-order in their total concentration, plotting of the second-order rate constant against the pH should give a bell-shaped curve.[85] The fact that this is not the case has been attributed to competition between two reactions, both leading to cleavage.[85] One of these is catalyzed by both the acidic and basic buffer constituent, whereas the other one is a simple general base catalyzed cleavage. The rate of the latter reaction is increased on decreasing the state of protonation of the buffer, and hence compensates for the rate retardation that the acid/base catalyzed reaction simultaneously experiences.

The results on the buffer catalyzed isomerization are even more complicated to interpret. As mentioned above, a rather inefficient buffer catalysis was observed for isomerization in imidazole buffers.[39] The second-order rate constant continuously increased with the increasing state of protonation of the buffer ([BH^+]/[$B]_{tot}$), referring to general acid catalysis. Unfortunately, these results were obtained without control of ionic strength. No buffer catalysis was observed in more basic morpholine buffers at a constant ionic strength.[87] By contrast, the isomerization was slightly retarded on increasing the buffer concentration. The catalysis by both of these buffers has recently been reinvestigated by controlling, not only the ionic strength, but also the medium effect of the uncharged buffer base, by using dioxane as their surrogate.[85] According to the results of these studies, the isomerization really is a general acid catalyzed reaction. However, this catalysis is not always visible, since the increasing concentration of the buffer base exerts an inhibitory effect. This inhibition becomes more pronounced on increasing the basicity of the buffer base. The origin of the inhibition is concurrent breakdown of the phosphorane intermediate to 2′,5′-NpN and a 2′,3′-cyclic phosphate, as described below in more detail.

The transesterification reactions of the phosphodiester bond seem to be rather sensitive to the medium effects, which together with the inefficiency of the buffer catalysis, makes reliable interpretation of the experimental results exceptionally difficult. As indicated above, several ambiguities related to determination of the catalysis type are of this origin. The first experimental evidence against a bell-shaped pH−rate profile was presented by Kirby and Marriot,[95] who showed that the cleavage of the 4-nitrophenoxymethyl ester of uridine 3′-phosphate (**13**) is



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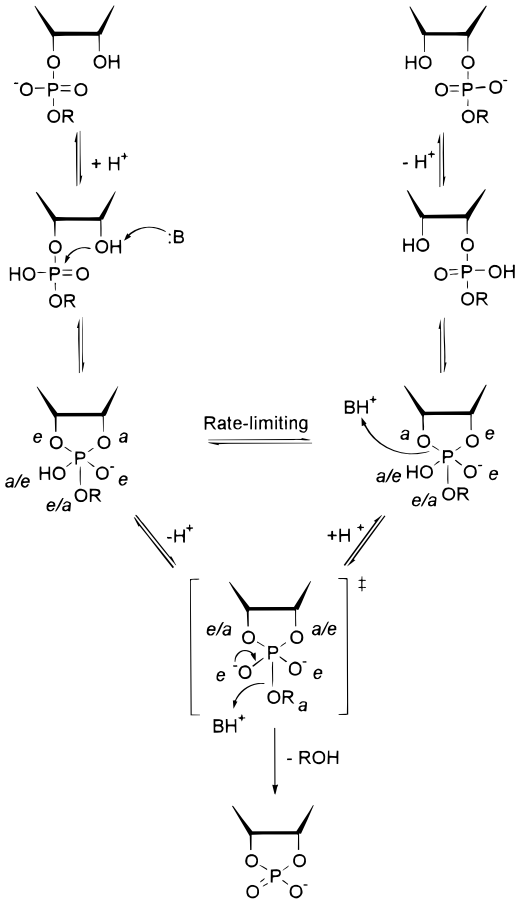
influenced by an unfavorable medium effect of free imidazole. Increasing imidazole concentration supresses the buffer-independent cleavage, which with this compounds in basic imidazole buffers is the predominant reaction. Consequently, the otherwise linear pH−rate profile becomes, at a constant buffer concentration, bell-shaped. A similar but less pronounced effect has been observed on the cleavage of 3′,5′-UpU on adding dioxane in the reaction solution.[85] Isomerization, in turn, seems to be simultaneously accelerated.

In summary, the cleavage of the 3′,5′-phosphodiester bond appears to proceed by two pathways in imidazole and morpholine buffers.[85] One of these is catalyzed by both the buffer acid and buffer base, but in such a manner that the reaction is only first-order in the total concentration of the buffer. The second reaction is catalyzed by the buffer base only. The isomerization, in turn, is catalyzed by the buffer acid, and inhibited by the buffer base.

C. Mechanism of the Imidazole/Imidazolium Ion (and Morpholine/Morpholinium Ion) Catalysis

On the basis of the observations discussed above, Breslow et al. have presented a mechanism for the cleavage and isomerization of the 3′,5′-phosphodiester bond by imidazole or morpholine buffers (Scheme 12).[39,85,90−93,96] The key argument behind this mechanism is that the cleavage must proceed via an intermediate, since it is (as well as the isomerization)

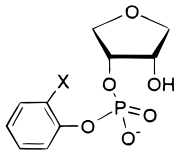
Scheme 12



first-order in total buffer concentration, but still both the buffer acid and buffer base serve as a catalyst. Moreover, the general acid and base catalysts have to act in different steps, one catalyzing the formation of the intermediate, and the other its breakdown. Another central argument is that this intermediate is common for the cleavage and isomerization. The underlying experimental observation is that a buffer base that catalyzes the breakdown of the intermediate to cleavage products, simultaneously inhibits isomerization. According to Breslow, the explanation is that the cleavage and isomerization proceed via the same intermediate, and the reactions branch at that stage. Since the breakdown of the intermediate to the cleavage and isomerization products competes with each other, increasing the mole fraction of the catalyst of the cleavage path, i.e., imidazole or morpholine, is reflected as less rapid isomerization. Hence it may also be concluded that the general base catalyzes the second step of cleavage, and the formation of the intermediate must then be catalyzed by a general acid.

Mechanistically the observed general acid catalysis for the formation of the phosphorane intermediate has been attributed to sequential specific acid and general base catalysis: the phosphodiester monoanion is protonated at a rapid preequilibrium, and the protonated phosphate is then attacked by the 2′-hydroxy function assisted by a general base. The intermediate is thus obtained as an isomer of the starting material, i.e., as a monoanion. This has been suggested to be required in order for the reaction to exhibit catalysis by both buffer constituents.[39,89] The general base catalyzed cleavage of the monoanionic intermediate to the 2′,3′-cyclic phosphate is, in turn, interpreted as sequential specific base and general acid catalysis: a rapid deprotonation to the phosphorane dianion is followed by general acid catalyzed departure of the 5′-linked nucleoside. Breakdown of the monoanionic intermediate to the isomerization products is assumed to proceed by rate-limiting pseudorotation. Accordingly, this step is uncatalyzed.

It is interesting to note that Oakenfull et al.[97] in mid-1960s suggested on the basis of their studies on cleavage of 3,4-dihydroxytetrahydrofuran 3-(phenyl phosphate) (**14a**) in imidazole and morpholine buffers



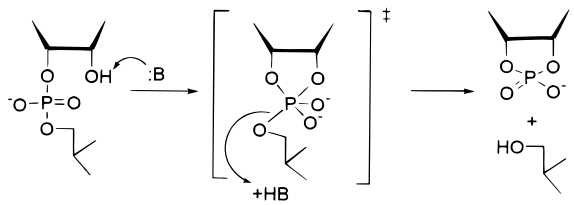
14a: X = H
14b: X = COO-

that the cleavage occurs via an intermediate. Furthermore, they noticed that the results obtained in morpholine buffers could not be divided by a standard method to contributions of general acid and base catalysis. At low state of protonation of the buffer ($[BH^+]/[B]_{tot}$), the reaction was apparently general acid catalyzed and and, at high state of protonation, general base catalyzed. In fact, these early suggestions are consistent with Breslow's mechanism.

As mentioned above, concurrent with the sequential general acid/base-catalyzed reaction (Scheme 12), a general base-catalyzed cleavage of the starting material has been suggested to take place.[85] Under conditions where the basic buffer constituent prevails, this process is actually the predominant buffer-dependent reaction. The reaction probably is a concerted process proceeding through a dianionic pentacoordinated species which is too short-lived to pseudorotate either as such or after protonation to monoanion (Scheme 13). It has been argued that this reaction is sensitive to changes in the ionic strength, which might explain the different shapes of the pH−rate profile obtained with and without the control of ionic strength.[85] This concerted reaction is the only buffer catalyzed process suggested to occur with substrates having a leaving group which is much better than a 5′-linked nucleoside.[95,98−100]

The mechanism depicted in Scheme 12 has also been criticized. One reason for the criticism[101,102] has been the somewhat uncontrolled conditions used in the original studies[39,88] that led to the proposal of sequential general acid/base catalysis. Originally the

Scheme 13

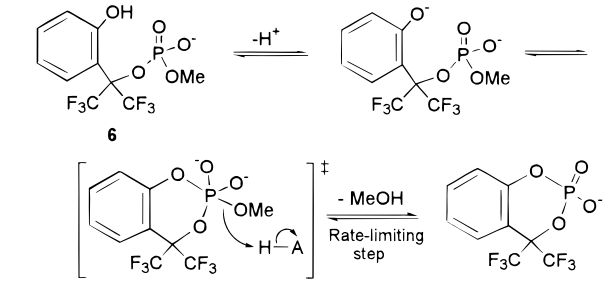


strongest evidence of this mechanism was, as discussed above, a bell-shaped pH−rate profile of the cleavage reaction. It was later shown by Breslow[85] that this actually was an artifact resulting from salt effects; on keeping the ionic strength constant, a linear dependence of rate on pH was observed at a constant total concentration of the buffer. Another subject of criticism has been the correction of the observed rate constants for the buffer-independent reaction competing with the buffer-catalyzed reactions. A simple subtraction was used for this purpose. Strictly speaking this is not correct since partial rate constants referring to buffer independent events appear in both the numerator and denominator of the expression of the observed rate constant.[101] Accordingly, it was questioned whether the inhibition of isomerization by the basic buffer constituent could simply result from unsuccessful correction for the buffer-independent reaction.[101] A considerable amount of additional experimental data has, however, recently been presented to show that the isomerization is really decelerated due to the general base catalyzed cleavage of the phosphorane intermediate to a 2′,3′-cyclic phosphate.[85,87,92,93]

Attention has also been paid[101−103] to the curious feature of the sequential bifunctional catalysis mechanism: that breakdown of the phosphorane intermediate is catalytically asymmetric. The monoanionic phosphorane undergoes general acid catalyzed cleavage of the P−O2′ and P−O3′ bonds, while the P−O5′ bond is cleaved by a similar mechanism only after deprotonation of the phosphorane to a dianion. This unexpected aspect of the mechanism is, however, consistent with the results obtained with uridine 3′-phosphotriester **5b**. The phosphorane derived form of this compound may be regarded as a model of the monoanionic phosphorane derived from 3′,5′-UpU. With this phosphorane, cleavage of the P−O2′ and P−O3′ bonds occurs much more readily than cleavage of the P−OMe bond, as discussed in section 2 in more detail.[55] Similarly, as also indicated above, the buffer-independent breakdown of the monoanionic intermediate derived from 3′,5′-UpU appears to favor the endocyclic cleavage.[40]

Investigations on cleavage of phosphodiesters having a well-defined rate-limiting step, either the formation or breakdown of the phosphorane intermediate, agree with Breslow's mechanisms. The cleavage of diester **14b** represents a case where the formation of the phosphorane intermediate may be assumed to be rate-limiting.[70] In weakly basic buffers, such as formate, acetate, and hydrogen phosphate, general acid catalysis was observed. A sequential specific acid and general base catalysis was proposed as the most plausible mechanistic interpretation,[70] consistent with the mechanism of Breslow (Scheme 12). The more basic TRIS buffer exhibited, in addition to this reaction, also mere general base catalysis: general base catalyzed attack of the neighboring hydroxy function on the monoanionic phosphodiester, now consistent with the mechanism in Scheme 13. Cyclization of **6** to a cyclic diester with concomitant departure of the methoxy group constitutes a model reaction for the rate-limiting decomposition of the phosphorane intermediate. The reaction of the monoanion of **6** was observed to be catalyzed by bases stronger than acetate ion (pK_a > 5.4).[56,104] The attacking nucleophile was concluded to be phenoxide ion, and hence the apparent base catalysis was attributed to kinetically equivalent general acid-catalyzed reaction of the dianion of **6**, exhibiting a Brønsted α of 0.33 (Scheme 14). According to this interpretation, the phosphorane dianion is a marginally stable, kinetically visible intermediate, not only a transition state. The departure of the leaving group takes place at an early transition state in concert with a proton transfer from the general acid. As discussed above, several experimental observations, however, suggest that the dianionic phosphorane is too unstable even to undergo protonation. Bearing this in mind, formation of the dianionic phosphorane can hardly be regarded as a clear preequilibrium step, but the general acid catalyzed departure of the leaving group rather takes place concerted with the attack of the phenoxide ion on phosphorus. Similarly, in Breslow's mechanism (Scheme 12) the dianionic phosphorane can hardly be regarded as a real intermediate, but breakdown of this species by general acid catalyzed departure of the leaving group, giving the cyclic phosphate, must efficiently compete with the reversal of its formation (i.e., with protonation of the dianionic phosphorane). Alternatively, the deprotonation of the monoanionic phosphorane is general base catalyzed, and the leaving group departs as alkoxide ion.

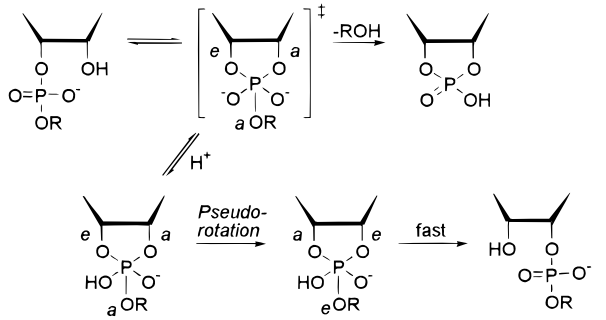
Scheme 14



Buffer catalysis has also been studied with **5b**.[55] The isomerization was observed to be catalyzed by the buffer base, as expected on the basis of the mechanism in Scheme 12. The additional alkyl group has taken the role of the specific acid catalyst. The catalysis is, however, rather modest, even so modest that it is difficult with full confidence to distinguish it from possible specific salt effects. The cleavage of **5b**, in turn, exhibits a clear general base catalysis, but this reaction cannot proceed by the pathway suggested for the cleavage of diesters, since the phosphorane intermediate does not carry a hydroxy proton that could be removed. The most plausible mechanism is specific base catalyzed formation of a monoanionic phosphorane, followed by general acid catalyzed cleavage of one of the methoxy ligands.

Perrin has suggested an alternative interpretation for the kinetic results of Breslow's group.[103] In this mechanism the formation of the intermediate is catalyzed by a general base that deprotonates the 2′-hydroxy function concerted with its attack on the monoanionic phosphodiester group. The dianionic

Scheme 15



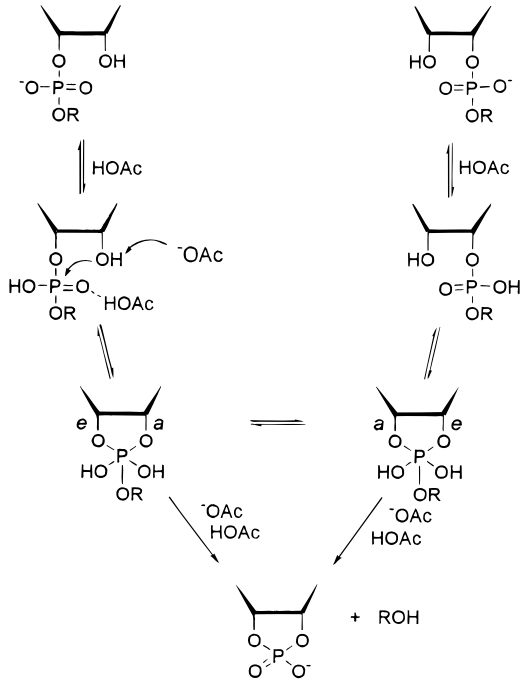
phosphorane obtained then undergoes general acid-catalyzed cleavage either to a 2′,3′-cyclic phosphate or back to the starting material (Scheme 15). Alternatively, one of the nonbridging oxygens of the dianionic intermediate is protonated, and the monoanionic phosphorane undergoes rate-limiting pseudorotation. The mechanism is consistent with the experimentally observed formal kinetics. The puzzling point of this mechanism is that the dianionic intermediate initially formed must be sufficiently long-lived to become protonated. As mentioned above, the experimental data available do not lend support to this assumption: a base-catalyzed isomerization that would be a definite proof for the feasibility of Perrin's mechanism has never been reported. Breslow[85] has underlined that Perrin's mechanism does not explain the observed catalysis by the acidic buffer constituent. Even in the case of isomerization that occurs by protonation of the dianionic intermediate, the protonation is fast and complete, and hence general acid catalysis should not be observed.

D. Catalysis by Carboxylate Buffer

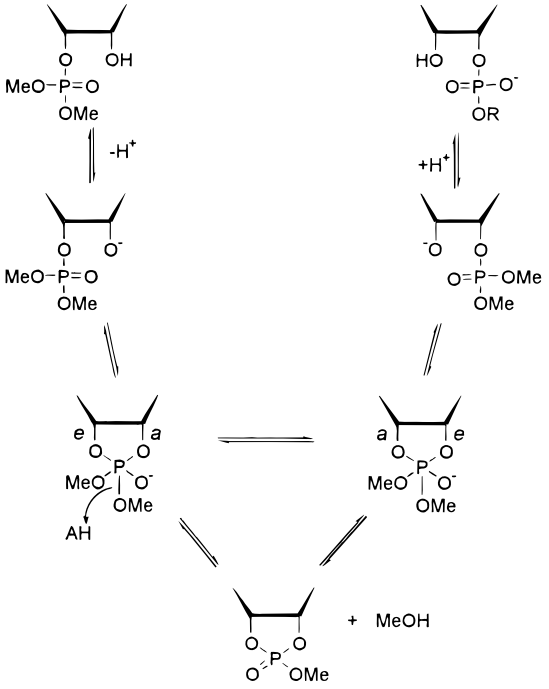
Buffers which are more acidic than imidazole or morpholine buffers also catalyze the cleavage and isomerization of the 3′,5′-phosphodiester bond, but the mechanism appears to differ from those depicted in Schemes 12 and 13. In acetic acid buffers, both the cleavage and isomerization show a second-order dependence of rate on the total concentration of the buffer, and only the buffer acid is catalytically active.[39,85] It has been tentatively suggested that one molecule of acetic acid is hydrogen bonded to the phosphoryl oxygen of neutral phosphodiester obtained by a rapid preequilibrium protonation, and the attack of the 2′-hydroxy function to this hydrogen bonded species is assisted by general base catalysis of acetate ion (Scheme 16). A neutral phosphorane is obtained, which subsequently either pseudorotates and undergoes the reversal of its formation, or is deprotonated to a monoanion and then converted to cyclic phosphate via general acid catalyzed cleavage of the P−O5′ bond.

By contrast, the studies with the triester analogue **5b** suggest a more straightforward mechanism.[55] The cleavage of **5b** in carboxylic acid buffers is first-order in the total concentration of the buffer, the catalytically active species being the buffer base. Since the buffer-independent isomerization under these conditions is much faster than the cleavage, it appears clear that the rate-limiting step of the cleavage is the

Scheme 16



Scheme 17



breakdown of the phosphorane intermediate. This, together with the fact that the buffer-independent reaction is initiated by the attack of 2′-oxyanion on phosphorus even at pH 3, suggests that the reaction occurs via specific base catalyzed formation and general acid catalyzed breakdown of the phosphorane intermediate (Scheme 17). The Brønsted β for the apparent general base catalysis is 0.7, which means that the Brønsted α for the general acid catalyzed reaction would be 0.3.[55] A similar mechanistic interpretation has been given for the general acid catalyzed cleavage/cyclization of the monoanion of **6**: intramolecular attack of oxyanion on neutral phosphate, followed by general acid catalyzed depar-

ture of the leaving group from the monoanionic intermediate.[56] Intrestingly, even the Brønsted α is very similar, viz. 0.27, suggesting an early transition state. Nunez and Nunez[105] have recently suggested that the bell-shaped pH−rate profile reported earlier[39] could be accounted for by occurrence of two competing mechanisms, viz. those in Schemes 13 and 17. However, Bresolw's evidence for the mechanism shown in Scheme 12 appears more extensive.

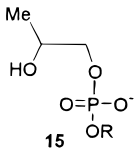
In most acidic carboxylic acid buffers, viz. formate and cyanoacetate buffers, the cleavage of **5b** becomes dependent on the concentration of the buffer acid.[55] In all likelihood, this reaction proceeds by formation of a neutral phosphorane, which undergoes general acid catalyzed decomposition to the cleavage products.

In summary, the general acid/base catalyzed cleavage and isomerization of the 3′,5′-phosphodiester bond form together with the simultaneously occurring buffer-independent reactions a most complicated reaction system. Undoubtedly, a lot more experimental results are needed before the mechanisms of all the partial reactions are on solid bases. For the time being, the mechanisms presented by Breslow must be regarded as at least a good working hypothesis, the central elements of which seem to withstand critical evaluation. Despite some criticism, no new experimental data that would definitely disagree with these mechanisms has been presented. The future, maybe, will show the final truth.

4. Structural Effects

A. The Effect of Leaving Group on the Cleavage via a Dianionic Pentacoordinated Transition State

The 3′,5′-phosphodiester bond is cleaved in aqueous alkali by the so-called in-line mechanism, as discussed in section 2. The attacking 2′-oxyanion and the departing 5′-oxyanion occupy the apical positions within a pentacoordinated structure, the lifetime of which is so short that it may rather be regarded as a transition state than a kinetically significant intermediate (Scheme 1). An analogous mechanism also operates in the general base catalyzed cleavage when the basic buffer constituent is a sufficiently strong base (Scheme 13). Since phenols are considerably stronger acids than aliphatic alcohols, and hence better leaving groups as oxyanions, nucleoside 3′-(aryl phosphates) (**2**; R = aryl**)** are cleaved by this mechanism much more readily than their alkyl conterparts (**2**; R = alkyl). Adenosine 3′-(phenyl phosphate) (**2**; R = Ph, B = adenine), for example, has been reported to be cleaved in aqueous alkali 10^5 times faster than 3′,5′-ApA.[106] Somewhat unexpectedly, the susceptibility of the aryl and alkyl esters to the basicity of the leaving group seems to differ considerably. The hydroxide ion catalyzed cleavage of uridine 3′-(alkyl phosphates) (**2**; R = alkyl, B = uracil) has been shown to be very susceptible to the basicity of the displaced alkoxide ion, the β_{lg} value being −1.28.[107] Comparable highly negative β_{lg} values have also been reported for other related intramolecular displacement reactions, such as cleavage of guanosine 3′-(benzyl phosphates) (β_{lg} = −0.9; **2**; R = substituted benzyl, B = guanine),[99,108] 2-hydroxypropyl alkyl phosphates (β_{lg} = −1.1; **15**; a recalculated value),[109] and the dianions of diesters **6** (β_{lg} = −1.13)[56] and **16** (β_{lg} = −1.26).[110] By contrast,



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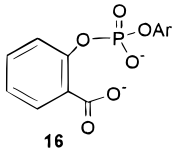

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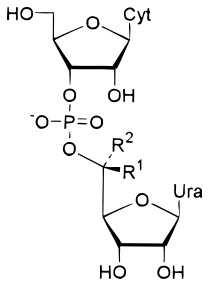
the cleavage of uridine 3′-(aryl phosphates) (**2**; R = substituted phenyl, B = uracil) is much less sensitive to the basicity of the leaving group, the β_{lg} value being −0.54 and −0.59 for the hydroxide ion and imidazole-catalyzed reactions, respectively.[99] These moderately negative β_{lg} values have been interpreted to indicate that both the PO2′ bond formation and P−OAr bond cleavage are only weakly advanced in the transition state, and the buildup of negative charge on the nonbridging phosphoryl oxygens on going from the initial to the transition state is rather modest.[99,111] While this may well be the case with aryl esters, the markedly negative β_{lg} values observed for the cleavage of the alkyl esters argue against this kind of a clearly concerted mechanism. As discussed by Dalby et al.,[56] the latter reactions rather proceed via a late transition state where the alkoxide ion character of the leaving group is well developed and the formation of the PO2′ bond is rather advanced. Accordingly, the dianionic phosphorane was regarded as a marginal intermediate, but having a lifetime too short for complete diffusional equilibration. One should still bear in mind that the bulk of the existing evidence argues against a dianionic oxyphosphorane intermediate that is sufficiently long-lived to undergo protonation to a phosphorane monoanion.[52]

Replacing either the *pro-R* or *pro-S* hydrogen with a methyl group at C5′ of the 5′-esterified nucleoside has only a moderate influence on the cleavage of the 3′,5′-phosphodiester bond, suggesting that the nucleophilic attack of the 2′-oxyanion is not highly susceptible to steric retardation.[112] Studies with the analogues of 3′,5′-CpU showed the cleavage rates the *R*- (**17a**) and *S*-isomers (**17b**) to be 14% and 9% of that of 3′,5′-UpU. Part of these rate retardations



17a: R^1 = H; R^2 = Me
17b: R^1 = Me; R^2 = H

may, however, result from replacing a hydrogen with a more electropositive methyl group, and from replacing the 3′-linked uridine with cytidine. The latter change has been shown to retard the alkaline cleavage up to 40%.[113] The extremely slow migration of

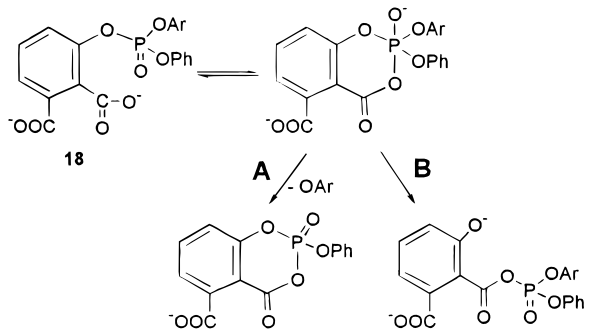
di-*tert*-butoxyphosphoryl group[114] compared to that of dimethylphosphoryl group,[55] however, clearly indicate that the steric factors are by no means negligible.

B. The Effect of Leaving Group on the Cleavage and Isomerization via a Phosphorane Intermediate

The studies of Dalby et al.[56] on diesters **6** nicely demonstrates the susceptibility of the cleavage of various ionic forms to the electronegativity of the leaving group. As mentioned above, the cyclization of the dianion of **6** is very susceptible to the basicity of the alkoxide ion displaced. However, as soon as the leaving group departs as an alcohol, the cleavage rate becomes rather insensitive to the polar nature of the leaving group, regardless of whether the departure starts from a monocationic, neutral, or monoanionic phosphorane (cf. Scheme 6). The β_{lg} values determined for these reactions are −0.15, −0.35, and −0.28, respectively.

Consistent with these studies, the cleavage of uridine 3′-(alkyl phosphates) is markedly insensitive to the electronegativity of the alkoxy group under acidic conditions, i.e., when the alkoxy group departs as an alcohol. The β_{lg} value for a reaction via a monocationic phosphorane (cf. Scheme 4) is only −0.12.[107] Electron withdrawal by a polar substituent, for example, undoubtedly weakens the P−OR bond by increasing its polarity, but simultaneously the protonation of the leaving oxygen atom is retarded. Evidently these effects almost completely cancel each other. The isomerization to 2′-(alkyl phosphates) shows a similar hardly noticeable dependence of rate on the electronegativity of the alkoxy group,[107] suggesting that the polar nature of the alkyl group remaining bonded to phosphorus upon breakdown of the phosphorane intermediate is of minor importance. The latter conclusion is consistent with the studies on intramolecular transesterification of phosphotriester **18**. This compound undergoes two alternative reactions via a common monoanionic pentacoordinated intermediate obtained by the attack of the 2-carboxylato anion on phosphorus (Scheme 18). Departure of the aryloxide ion gives a cyclic phosphotriester (reaction **A**), while rupture of the endocyclic PO bond leads to an acyclic triester (reaction **B**). Reaction **A** is, as with diesters, very susceptible to the basicity of the leaving aryloxide ion, the β_{lg} being −1.44.[115] The highly negative β_{lg} value was taken as an indication of a stepwise mechanism,[115] although more recent studies suggest that with phosphotriesters the departure of phenoxide leaving groups is rather a concerted process.[116] The rate of the endocyclic cleavage (reaction **B**) is also influenced by polar substituents on the aryl group, but to a much lesser extent. The β value for this structural effect equals −0.30.[115]

Scheme 18



The transesterification reactions of nucleoside 3′-(aryl phosphates) appear to differ from those of 3′-(alkyl phosphates), not only by absolute reaction rates but by relative reactivities of various ionic forms. In striking contrast to 3′,5′-UpU, transesterification to uridine 2′,3′-cyclic phosphate is the predominant reaction of uridine 3′-(2-chlorophenyl phosphate) (**2**; R = 2-ClPh, B = uracil) over the entire pH range.[49] Clear evidence of phosphate migration has been obtained only at pH < 2, and even then the isomerization to the 2′-(aryl phosphate) is considerably slower than the cleavage, representing 10% of the total disappearance of the starting material at pH 2 and 25% at pH 1. The cleavage reaction shows in the pH range 0−8 three kinetically distinct terms: first-order dependence on hydronium ion concentration at pH < 2, pH independence at pH 2.5−5.5, and first-order dependence on hydroxide ion concentration at pH > 6. The pH−rate profile reported earlier for the buffer-independent cleavage of 3,4-dihydroxytetrahydrofuran 3-(phenyl phosphate) (**14a**) is qualitatively similar, except the fact that second-order dependence of rate on hydronium ion concentration is observed in very acidic solutions.[57]

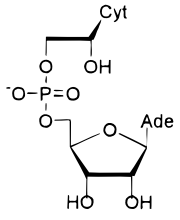
The reaction via a neutral phosphorane intermediate, i.e., the reaction which is first-order in hydronium ion concentration (cf. Scheme 7), is with uridine 3′-(2-chlorophenyl phosphate) about 200 times as fast as with 3′,5′-UpU.[40,49] However, the difference between the observed cleavage rates is much smaller, since the predominant reaction of 3′,5′-UpU is at pH < 2 the one via a monocationic phosphorane (cf. Scheme 4). Evidently the latter reaction is not much more facile with aryl esters than with the alkyl esters, and hence the reactivity difference gradually disappears on going to concentrated acidic solutions (pH ≈ 0).

The pH-independent cleavage of uridine 3′-(2-chlorophenyl phosphate) is 4 orders of magnitude faster than that of 3′,5′-UpU,[40,49] and more than 2 orders of magnitude faster than the pH-independent mutual isomerization of 2′5′- and 3′,5′-UpU. In all likelihood, this reaction proceeds by the departure of 2-chlorophenoxide ion from the monoanionic phosphorane intermediate obtained by the attack of 2′-oxyanion on neutral phosphate. It is worth noting that while the specific and general base catalyzed cleavage of nucleoside 3′-(aryl phosphates) undoubtedly is a concerted reaction, as discussed above, the pH-independent cleavage may still occur via a monoanionic intermediate. Obviously the 2-chlorophenoxide ion is such a good leaving group that pseudorotation and subsequent departure of the 3′-oxyanion cannot compete with the cleavage.

The preceding comparisons clearly show that the transesterification reactions of nucleoside 3′-(aryl phosphates) differ in several respects from those of the 3′-(alkyl phosphates), including 3′,5′-NpN. Accordingly, one may well question whether the aryl esters are appropriate model compounds for the mechanistic studies of internucleosidic phosphodiester bonds of RNA. While accurate kinetic data is undoubtedly obtained much more conveniently than with dinucleoside monophosphates, care must be exercised on extrapolating the results of such studies to ribonucleoside esters having a worse leaving group.

C. The Effect of Sugar Moiety Structure

Epimerization of the 3′-phosphorylated nucleoside at C2′ (*ribo* to *arabino*) entirely prevents the nucleophilic attack of the 2′-hydroxy function on phosphorus.[57] Even replacing of the sugar ring with an acyclic diol markedly retards both the cleavage and isomerization.[117] The hydroxide ion catalyzed cleavage of 3′,5′-UpA, for example, is 500 times as fast as that of its acyclic 1-[(2′*S*)-2′,3′-dihydroxypropyl]cytosine analogue **19**. The acid catalyzed cleavage and



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isomerization are too slow to compete with depurination of the adenosine moiety. This means that the rate retardation must be greater than 1 order of magnitude.

While the neighboring hydroxy function can attack on the phosphorus of ribonucleoside 3′-phosphodiesters only from *cis*-position, the situation appears to be different with pyranoid phosphoesters. Both *cis*- and *trans*-hydroxy groups may attack on phosphorus, although the attack from the *cis*-position is considerably faster.[118] Consistent with these observations, low-temperature NMR spectroscopic measurements on the equilibration of phosphoranes **20a** and **21a** (Chart 2) with the corresponding phosphonium ions **20b** and **21b** suggest that a phosphate and hydroxy group as vicinal *cis*-substituents on a five-membered ring have a particularly suitable orientation for phosphorane formation.[119]

Chart 2

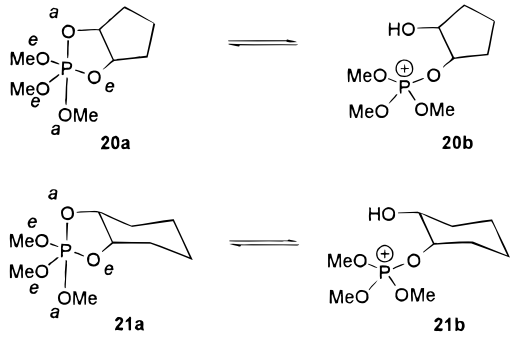
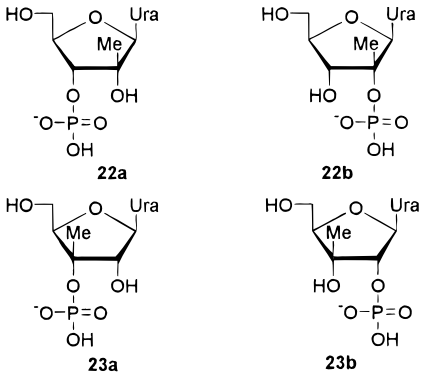


Table 1. Observed Pseudo-First-order Rate Constants (k_{obs}) for the Hydroxide Ion Catalyzed Cleavage of 3′,5′- and 2′,5′-NpN in Aqueous Sodium Hydroxide, Kinetically Determined pK_a Values of the 2′- or 3′-Hydroxy Function, and the First-Order Rate Constants (k_O^-) for the Cleavage of the 2′/3′-Oxyanions of the Starting Materials[a]

compd	k_{obs}, 10^{-3} s^{-1} [b]	pK_a	k_O^-, 10^{-3} s^{-1}
2′,5′-UpU	1.72	12.84[c]	12.8
3′,5′-UpU	1.61	12.55[d]	6.92
2′,5′-UpA	1.98	12.97[c]	18.8
3′,5′-UpA	1.71	12.52[d]	7.41
2′,5′-ApU	0.93	12.73[c]	5.13
3′,5′-ApU	0.96	12.04[d]	2.00
2′,5′-ApA	1.32	12.70[c]	7.38
3′,5′-ApA	0.72	12.24[d]	5.68

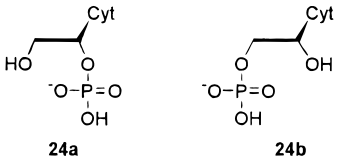
[a] Taken from ref 40. The data refer to 60 °C at $I = 1.0$ mol L^{-1}. [b] In 0.1 mol L^{-1} aqueous sodium hydroxide at $I = 1.0$ mol L^{-1}. [c] pK_a of the 3′-OH. [d] pK_a of the 2′-OH.

Chart 3



The 2′,5′-phosphodiester bond is cleaved approximately as rapidly as the 3′,5′-bond both in aqueous acid and alkali,[40,41] and the isomerization of a 2′,5′-bond to a 3′,5′-bond is as fast as its reverse reaction. The general acid/base catalyzed transesterifications show a hardly detectable difference in rate, the 3′,5′-bond being cleaved slightly faster and isomerized slightly less rapidly than the 2′,5′-bond.[39] The 2′-hydroxy function of 3′,5′-NpN is about 0.5 pK_a unit more acidic than the 3′-hydroxy group of 2′,5′-NpN (Table 1).[40] The 3′-oxyanion is, in turn, about twice as efficient nucleophile as the 2′-oxyanion, and hence the cleavage rates are at pH < 12 almost equal.

As discussed above, the phosphate migration between the two secondary hydroxy groups of unmodified ribonucleosides is equally as fast in both directions. If one of the participating hydroxy functions is either primary or secondary, the situation is different. Studies with 3′- and 2′-phosphates of 2′-(**22a**,**b**) and 3′-*C*-methyluridine (**23a**,**b**) (Chart 3) have shown that the migration from a tertiary hydroxy to a secondary one is 1 order of magnitude faster than the migration between two secondary hydroxy groups.[120] Studies on the interconversion of 1-[(2′S)-2′,3′-dihydroxypropyl]cytosine 2′- and 3′-*O*-phosphates (**24a**,**b**) have, in turn, shown that the migration from a secondary to primary hydroxy group



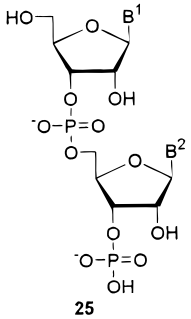
24a **24b**

is 3 times as fast as its reverse reaction,[117] but still slower than the migration between the secondary hydroxy groups in ribonucleosides.

D. The Effect of Base Moiety

The early studies of Witzel showed that the base moiety structure has only a modest effect on the cleavage of the 3′,5′-phosphodiester bond;[113] among the eight dinucleoside monophosphates (3′,5′-NpA and 3′,5′-NpC; N = A, C, G, U) studied by him, the most reactive dimer, 3′,5′-UpC, was cleaved in aqueous acid 3 times as rapidly as the least reactive one, 3′,5′-GpA (Table 2). More recent studies have given similar results and shown that the isomerization is as insensitive to the base moiety structure as the cleavage.[40] Indeed, very few generalizations can be done. The slightly higher reactivity of pyrimidine nucleoside 3′-phosphodiesters compared to their purine counterparts was orginally interpreted as an indication of intramolecular participation of the pyrimidine O^2 atom,[113] but this suggestion has not been accepted.[44,121]

Table 3 summarizes the kinetic data for the hydroxide ion catalyzed cleavage of 3′,5′-NpN and their 3′-phosphates (3′,5′-NpNp, **25**). The effect of the base



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moiety structure is slightly greater than under acidic conditions.[40,113,122] It has been suggested that intramolecular base stacking would largely determine the reactivity order of dinucleoside mono- or diphosphates. Comparison of the kinetic data with the theoretically calculated mole fractions of the stacked form of each 3′,5′-NpN,[123] however, reveals that if any such correlation exists, it must be a very approximate one.

Table 2. Observed Pseudo-First-Order Rate Constants for the Cleavage (k_{cl}) and Isomerization (k_{is}) of 3′,5′-NpN

	k_{cl}, 10^{-3} s^{-1}		k_{is}, 10^{-3} s^{-1}	
compd	*a*	*b*	*c*	*d*
3′,5′-UpU	19.0		29.0	0.0013
3′,5′-UpC		0.077		
3′,5′-CpC		0.055		
3′,5′-GpC		0.031		
3′,5′-ApC		0.035		
3′,5′-ApU	11.3		10.4	0.0012
3′,5′-UpA	20.0	0.064	10.8	0.0015
3′,5′-CpA		0.046		
3′,5′-GpA		0.026		
3′,5′-ApA	15.0	0.030	10.3	0.0010

[a] In 1.0 mol L^{-1} HCl(aq) at 90 °C.[40] [b] In 0.3 mol L^{-1} HCl(aq) at 45 °C.[113] [c] In 1.0 mol L^{-1} HCl(aq) at 90 °C.[40] [d] For pH-independent isomerization at 90 °C.[40]

Table 3. Observed Pseudo-First-Order Rate Constants for the Hydroxide Ion Catalyzed Cleavage of 3′,5′-NpN and 3′,5′-NpNp[a]

3′,5′-NpN	k, 10^{-5} s^{-1} [b]	3′,5′-NpNp	k, 10^{-5} s^{-1} [c]
3′,5′-UpA	15.7	3′,5′-UpGp	6.47
3′,5′-ApA	5.3	3′,5′-CpGp	3.87
3′,5′-GpA	7.3	3′,5′-GpUp	3.03
3′,5′-CpA	12.5	3′,5′-ApUp	2.09
3′,5′-ApC	4.8	3′,5′-ApGp	2.01
3′,5′-GpC	8.9	3′,5′-ApCp	1.90
3′,5′-CpC	12.6	3′,5′-ApAp	1.53
3′,5′-UpC	22		

[a] See also the data in Table 1. [b] In 0.5 mol L^{-1} NaOH(aq) at 28 °C.[113] [c] In 0.2 mol L^{-1} NaOH(aq) at 25 °C.[122]

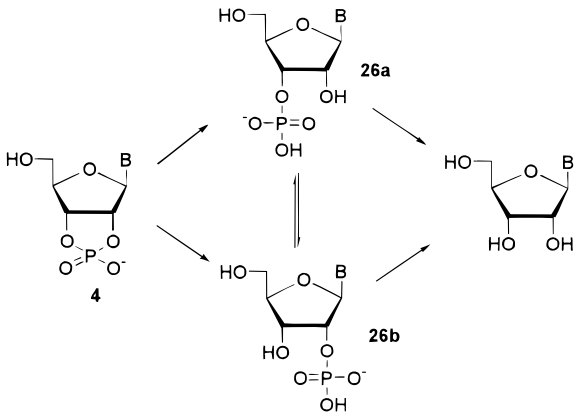
Although the base moiety does not markedly influence on the reactions of the phosphoester moiety, it plays an important role by participating in several competing reactions, such as depurination or deamination. Acid-catalyzed depurination competes with the phosphoester transesterification of purine nucleoside 3′-phosphodiesters.[40,41] Since the depurination is first-order in hydronium ion concentration,[124] while the phosphoester reactions tend to exhibit a second-order dependence of rate on acidity, the depurination competes most efficiently with the phosphoester cleavage under mildly acidic conditions (pH 2−5). Another significant side reaction is the hydrolytic deamination of the cytosine base to uracil. This reaction is catalyzed by buffer acids and bases in addition to hydronium and hydroxide ions.[125−128] The uncatalyzed deamination is also sufficiently rapid to compete with the buffer-independent phosphoester transesterifications under neutral conditions.[48,128] Accordingly, deamination is most likely encountered as a side reaction in buffered solutions at pH 4−7.[48] The base moiety reactions of RNA have been previously reviewed in more detail.[129]

5. Reactions of 2′,3′-Cyclic Phosphates

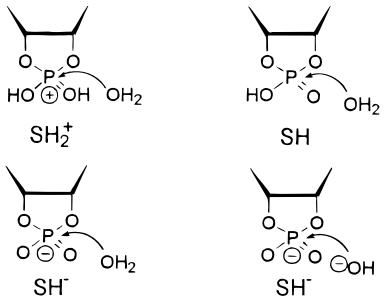
A. Hydrolysis of Nucleoside 2′,3′-Cyclic Phosphates

The cleavage of internucleosidic 3′,5′-phosphodiester bonds gives 2′,3′-cyclic phosphates (**4**) as initial products. These compounds are then hydrolyzed to a mixture of nucleoside 2′- (**26b**) and 3′-phosphates (**26a**) (Scheme 19).[43−47] The reaction shows four distinct kinetic terms, viz. the dependence of rate on $[S^-][H^+]^2$, $[S^-][H^+]$, $[S^-]$, and $[S^-][OH^-]$.[44−47] Here S^- denotes the monoanionic form of the cyclic phosphate that predominates over the entire pH range. No deviation from a second-order dependence of rate on $[H^+]$ could be observed even at pH < 1, which suggests that the cyclic phosphodiesters are more acidic than 3′,5′-NpN which have $pK_a \approx 1$.[45,46] It is worth noting that the kinetic terms are the same as

Scheme 19



Scheme 20

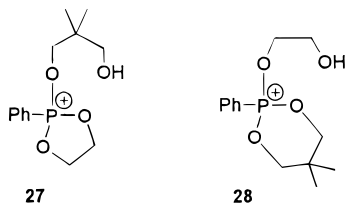


involved in the cleavage of 3′,5′-NpN at pH < 10. Each of the partial reactions is, however, faster than the corresponding reaction of 3′,5′-NpN. For example, the reactions referring to dependence of rate on $[S^-][H^+]^2$, $[S^-][H^+]$, $[S^-]$, and $[S^-][OH^-]$ are with adenosine 2′,3′-cyclic phosphate (**4**, B = adenine) 100, 300, 80, and 10 times as fast as with 3′,5′-UpU.[40,46] On using 3′,5′-NpN as a starting material, intermediary accumulation of 2′,3′-cyclic phosphate is hence detected only under alkaline conditions, and even then with difficulty. The 3′-phosphate predominates among the hydrolysis products under acidic and neutral conditions, the 2′- and 3′-phosphates being formed in approximately 1:2 ratio.[46,47] In alkaline solutions, the product distribution between the isomeric phosphates is more even, though the 3′-phosphate still prevails.[47,120,130,131]

The partial reactions indicated above most likely proceed by an attack of a water molecule on either monocationic (SH_2^+), neutral (SH), or monoanionic (S^-) starting material, and by an attack of hydroxide ion on the monoanionic species (Scheme 20). The attack of a water molecule on any ionic form of the starting material may be assumed to give a pentacoordinated intermediate having either the O2′ or O3′ in an apical position. Deprotonation of the phosphorus-bonded water molecule and protonation of the apical sugar oxygen then lead to formation of nucleoside 2′- and 3′-phosphates. The reaction of the anionic starting material with hydroxide ion may, in turn, be assumed to proceed by a direct in-line displacement of either of the 2′- and 3′-oxyanion via a pentacoordinated transition state.

Hydrolysis of the 2′,3′-cyclic phosphates is also catalyzed by general acids and bases.[44,45] In contrast to the cleavage of 3′,5′-NpN, the reaction is second-order in the total concentration of the buffer. Both imidazole and imidazolium ion serve as a catalyst, the former being 3 times as efficient catalyst as the latter.[45] The second-order dependence of rate on imidazole concentration suggests that imidazole and imidazolium ion are involved in the same transition state, serving as a general base and acid, respectively. Consistent with such a highly ordered transition state, the activation entropy for the imidazole catalysis has been observed to be as negative as -210 J K^{-1} s^{-1}, and the reaction shows a deuterium solvent isotope effect of 3.8. Catalysis by acetate, cacodylate, phosphate, and borate ions has also been reported, the Brønsted β being 0.4.[45]

The origin of the enhanced hydrolysis of five-membered cyclic phosphoesters compared to their acyclic counterparts has been the subject of considerable interest during the past two decades.[132] Evidence for the importance of both the ring strain[132,133] and stereoelectronic effect[81,134,135] has been presented. Recent quantum chemical calculations attribute the rate acceleration to different solvation of the transition states formed from cyclic and acyclic substrates.[62,65,74,136] Studies with a (5,6)-spirophosphorane suggest that the ring strain is not, at least completely, relieved on going from a five-membered cyclic phosphate to a phosphorane: the Gibbs free energy of a five-membered cyclic phosphonium ion (**27**) still is 10−20 kJ mol^{-1} higher than, for example, that of its six-membered counterpart (**28**).[137]



2′,3′-Cyclic phosphates (**4**) are hydrolyzed about 1 order of magnitude faster than ethylene phosphate (**12**),[43,138] the reaction being enthalpically more favored.[139,140] Whether this increased instability results from extra ring strain that results from fusion of the five-membered phosphodiester ring with the ribofuranose ring and is released on going to the pentacoordinated intermediate remains an open question. Anyway, it is interesting to note that opening of the ribofuranose ring decelerates the hydrolysis: the acyclo nucleoside cyclic phosphate **29** is hydrolyzed in aqueous alkali 1 order of magnitude more slowly than cytidine 2′,3′-cyclic phosphate (**4**; B = cytosine).[117] The product having the secondary hydroxy function phosphorylated initially predominates. Respectively, 2′- and 3′-*C*-methyluridine 2′,3′-cyclic phosphates (**30a**,**b**) are hydrolyzed predominantly to products having the more substi-

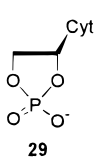


Table 4. Rate Constants for Hydronium Ion Catalyzed Hydrolysis of Nucleoside 2′,3′-Cyclic Phosphates

compd	*T*, °C	*I*, mol L^{-1}	k_H, L^2 mol^{-2} s^{-1}	ref
2′,3′-cUMP	50	0.1	1.1[a]	44
	50	1.0	0.58	45
	90	0.1	16.1	47
2′,3′-cCMP	50	0.1	0.79	117
	30	0.1	0.17	44
2′,3′-cAMP	30	0.1	0.066	44
	90	0.1	7.6[b]	46

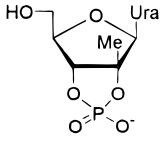
[a] Calculated from the temerature dependece of k_H determined at 25−40 °C. The activation parameters (at 25 °C) calculated from the same data are: $\Delta H^{\ddagger} = 58 \pm 8$ kJ mol^{-1} and $\Delta S^{\ddagger} = -64 \pm 27$ J K^{-1} mol^{-1}. [b] $\Delta H^{\ddagger} = 63 \pm 1$ kJ mol^{-1} and $\Delta S^{\ddagger} = -49 \pm 3$ J K^{-1} mol^{-1} at 25 °C.

Table 5. Rate Constants for the Hydroxide Ion Catalyzed Hydrolysis of Nucleoside 2′,3′-Cyclic Phosphates

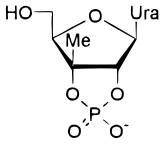
compd	*T*, °C	*I*, mol L^{-1}	k_{OH}, L mol^{-1} s^{-1}	ref
2′,3′-cUMP	50	0.1	0.018[a]	44
	50	0.1	0.016	120
	90	0.1	0.18	47
2′,3′-cCMP	50	0.1	0.032	117
	40	0.1	0.0069	44
2′,3′-cAMP	90	0.1	0.7	46

[a] Calculated from the temperature dependece of k_{OH} determined at 25−40 °C. The activation parameters (at 25 °C) calculated from the same data are: $\Delta H^{\ddagger} = 56 \pm 11$ kJ mol^{-1} and $\Delta S^{\ddagger} = -106 \pm 35$ J K^{-1} mol^{-1}.

tuted (in this case tertiary) hydroxy function phosphorylated.[120] Evidently the hydroxy function on less



30a



30b

substituted carbon is more apicophilic, and hence departs more readily. The base moiety has only a minor effect on the hydrolytic stability nucleoside 2′,3′-cyclic phosphates both in aqueous acid and alkali (Tables 4 and 5). Deamination of the cytosine moiety competes with the hydrolysis of 2′,3′-cCMP under neutral conditions.[44]

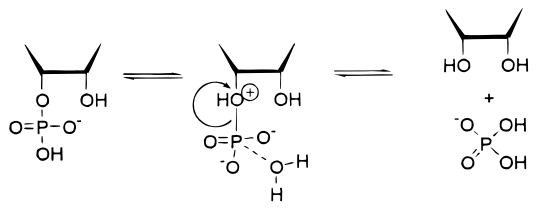
B. Interconversion and Dephosphorylation of Nucleoside 2′- and 3′-Phosphates

The 2′- and 3′-phosphates obtained as the hydrolysis products of 2′,3′-cyclic phosphates undergo two concurrent reactions, as known previously from early 1950s: interconversion and hydrolytic dephosphorylation (Scheme 19).[12,15] Accordingly, the cleavage of the internucleosidic phosphodiester bonds may give complicated product mixtures containg a 2′,3′-cyclic phosphate, 2′- and 3′-phosphates and unphosphorylated nucleoside, even if the possible side reactions at sugar or base moiety are not taken into account. The phosphate migration closely resembles the mutual isomerization of ribonucleoside 2′- and 3′-phosphodiesters.[46,48] Even the rates of these two reactions are comparable, as long as either the neutral or anionic ionic form predominates, i.e., at pH < 6. In other words, replacing the 5′-linked nucleoside of 3′,5′-NpN with hydrogen does not dramatically affect the rate or mechanism of the isomerization reaction. The situation is different at pH > 6. The predominant ionic form of nucleoside 2′/3′-phosphates is dianion under these conditions. The extra negative charge shields the phosphate group against the intramolecular attack of the 2′-oxyanion, and hence the phosphate migration is retarded, in striking contrast to the isomerization of diesters. The reaction from 2′- to 3′-phosphate is over the entire pH range faster than its reverse reaction, the ratio of the rate constants varying from 2.1 to 2.6.[46]

While nucleoside 2′- and 3′-phospates are in all likelihood hydrolyzed to a free nucleoside by a dissociative mechanism, as phosphate monoesters in general (see the discussion in next paragraph), their mutual isomerization seems to proceed by an associative mechanism, i.e., via initial attack of the neighboring hydroxy function. The reaction does not, however, proceed by intermediary formation of a cyclic phosphate; the incorporation of ^{18}O from $^{18}H_2O$ into adenosine 2′- and 3′-phosphates is much slower than the interconversion of these compounds.[46] The isomerization differs in this respect from that of acyclic compounds: a considerable part of phosphate migration between the vicinal hydroxy groups of glycerol[141] and 1,2-propanediol[142] has been shown to proceed via a cyclic diester. Evidently the cyclization of a phosphomonoester to a five-membered cyclic phosphate is more difficult with nucleosides than with acyclic compounds. This kind of cyclization has clearly been observed only with **22b** and **23a**.[120] In this case about 40% of the migration proceeds via the cyclic phosphate.

The neighboring hydroxy function does not appear to participate in the hydrolytic dephosphorylation of ribonucleoside 2′- and 3′-monophosphates to nucleosides.[46,48] As with simple alkyl phosphates, the reactive ionic form is the substrate mononion.[143] Accordingly, the dephosphorylation exhibits a broad pH maximum at pH 3−5. Under these conditions, the reaction is slightly faster than the interconversion of 2′- and 3′-phosphates. The mechanism of the hydrolysis of phosphomonoesters has been discussed elsewhere,[143−147] very recently by Hengge et al.[148] and Wilkie and Gani.[149] The essential features of the reaction include a proton transfer from the phosphate hydroxy ligand to the esterified oxygen, either directly or through water molecules, and subsequent rupture of the PO bond concerted with nucleophilic attack of a molecule of water (Scheme 21).

Scheme 21



6. The Cleavage and Isomerization of the Phosphodiester Bonds of Polyribonucleotides

The pH−rate profiles for the cleavage and isomerization of the phosphodiester bonds of poly(U) have been determined by following the progress of the reactions by enzymatic digestion with RNase A and subsequent RP HPLC.[42] The profiles show the same kinetic terms as those determined earlier for 3′,5′-NpN. However, one difference is worth noting. Both the cleavage and isomerization are under acidic conditions up to 1 order of magnitude faster with poly(U)[42] than with 3′,5′-UpU.[40] By contrast, under alkaline conditions no marked difference between the stabilities of the 3′,5′-bonds of polymeric and dimeric starting materials is seen. It has been tentatively suggested that the hydronium ion catalyzed partial reactions are abnormally fast with polymers, owing to enhanced protonation of the phosphodiester groups of polyribonucleotides. The observed rate acceleration would thus be of electrostatic origin. Consistent with this proposal, the cleavage of nonterminal phosphodiester bonds has been reported to be favored over that of terminal bonds in aqueous acid, but not in aqueous alkali.[42] Already the early studies on RNA hydrolysis led to observations consistent with those discussed: the reaction order with respect to hydronium ion was shown to be between 1 and 2,[150] while the alkaline hydrolysis was observed to be first-order in hydroxide ion concentration.[150,151] DMSO has been shown to accelerate markedly the alkaline cleavage.[152]

The cleavage of the internucleosidic phosphodiester bonds of poly(U) gives at pH 5 oligomeric products that predominantly bear a 3′-terminal 2′,3′-cyclic phosphate group, and on prolonged treatment products having an unphosphorylated 3′-end start to accumulate.[42] These results indicate that while transesterification of the 3′,5′-bonds to 2′,3′-cyclic phosphates is accelerated at polymeric level, the subsequent hydrolysis of the 3′-terminal cyclic phosphates is not faster than with monomeric starting materials. Accordingly, hydrolysis of the terminal 2′- and 3′-phosphates efficiently competes with their formation. Among the terminal monophosphates, 3′-phosphates predominate over the 2′-phosphates, consistent with the hydrolysis of monomeric nucleoside 2′,3′-cyclic phosphates.

It is worth noting that the acid-catalyzed isomerization of the 3′,5′-phosphodiester bonds to the 2′,5′-bonds is also at the polymeric level approximately as fast as the chain cleavage.[42] This fact seriously hampers the chemical synthesis of oligoribonucleotides, since an oligomer containing an 2′,5′-bond is extremely difficult to separate from the desired all-3′,5′-oligomer. The isomerization is extremely rapid as long as the phosphodiester bond is protected.[54,55] It was shown that substantial phosphate migration took place when synthetic uridine oligoribonucleotides protected with 2′-*O*-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl] group (2′-*O*-Fpmp) were subjected to acidic conditions for Fpmp deprotection.[153] The same observation has been performed earlier on removing 2′-protecting groups from dinucleoside monophosphates under acidic or neutral conditions.[154−159]

Consistent with the original observations of Breslow's group on the cleavage of poly(U),[89,160] the cleavage of $tRNA^{Asp}$ has been shown to be first order in buffer concentration in several molar imidazole buffers.[161] In 2 mol L^{-1} buffer, a bell-shaped dependence of rate on pH was observed. The cleavage predominantly took place within the single-stranded regions. Interestingly, in controls where imidazole was replaced with HEPES, which is as basic as imidazole, the cleavage of was negligible. Possibly the enhanced catalytic influence of imidazole buffers may be accounted for by electrostatic interaction between the imidazolium ion and the polyanionic substrate. The HEPES buffers are, in turn, made of an anionic and neutral (zwitterionic) species. Possibly, the observation of Breslow's group, according to which imidazole catalysis is clearly seen with poly(U),[89] whereas with 3′,5′-UpU the specific catalysis efficiently competes with imidazole catalysis,[39] is of the same origin. Alternatively, a differing medium effect may be responsible for the differences observed between the two buffer systems, given the high concentrations of buffer species used.

The double-helical RNA undergoes phosphodiester bond cleavage much more reluctantly than a random coil. This has been suggested to be in particular the case with a right-handed antiparallel double helix,[162,163] and explained to result from the double-helical structure preventing the entering 2′-hydroxy function and the departing 5′-oxygen to occupy simultaneously the required apical position in the phosphorane intermediate. By contrast, a 2′,5′-linkage when engaged in a right-handed double-helix has been shown to be cleaved almost 3 orders of magnitude faster than a 3′,5′-bond.[164] The structure and reactivity of double stranded RNA has been recently reviewed.[163] Small synthetic lariat-type oligoribonucleotides have been shown to undergo site-specific cleavage at a single phosphodiester bond with a tetra- or pentanucleotide loop.[165]

7. Cleaving Agents. Toward Artificial Nucleases by Proton-Transfer Catalysis

The desire to prepare artificial RNases that would sequence selectively cleave the mRNA encoded by a certain gene, and hence inhibit the gene expression, has directed the interest toward polyfunctional catalysts that would efficiently cleave the RNA phosphodiester bonds.[37,38] Most of such cleaving agents are based on metal ions, and they are described elsewhere in this issue. Purely organic multifunctional catalysts have, however, also shown some promise. These catalysts have been studied, not only as a potential basis for artificial RNases, but also as mimetics with which to elucidate in detail the elementary processes taking place at catalytic centers of protein enzymes.

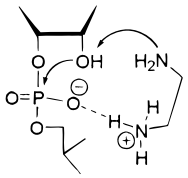
A. Aliphatic Di- and Polyamines as Cleaving Agents

The simplest bifunctional catalysts that have been shown to cleave the 3′,5′-phosphodiester bonds are

α,ω-alkanediamines. They accelerate the cleavage of 3′,5′-ApA from one to 2 orders of magnitude more efficiently than the corresponding monoamines.[166,167] They also degrade poly(A), poly(U), and poly(C), but not poly(G), to fragments shorter than pentamers.[168] 1,2-Ethylenediamine and its *N*-methyl and *N*,*N*′-dimethyl derivatives have been reported to be more efficient catalysts than methylenediamine, longer α,ω-alkanediamines, or oligoamines, such as diethylenetriamine, triethylenetetraamine or *N*,*N*′-bis-(aminoethyl)-1,3-propanediamine.

Both a neutral diamine and its monocation have been suggested to serve as a catalyst, the neutral species being more effective. The predominant partial reaction under neutral conditions is, however, the one catalyzed by the monocation, since the mole fraction of the neutral species is too small to allow a significant contribution. The catalysis has been proposed to involve hydrogen bonding of the ammonium ion center to the negatively charged phosphodiester group and general base catalyzed removal of proton from the attacking 2′-hydroxy function by the neutral amino group of the same catalyst molecule (Scheme 22).[167] Hydrogen bonding to the

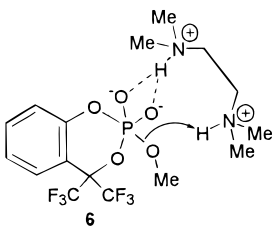
Scheme 22



phosphodiester group receives support from the fact that trimethylation of one of the amino functions reduces the catalytic efficiency by a factor of 4.4, although electrostatic interaction between the anionic phosphodiester group and the quaternary ammonium ion center would still be possible. Consistent with the partially rate-limiting proton transfer, the monocation-catalyzed reaction shows a solvent isotope effect $k(H_2O)/k(D_2O) = 2.0$. This mechanistic interpretation is essentially similar to that presented earlier by Kirby's group for the diamine dication catalyzed cyclization/cleavage of **6** [56,104] and the diamine monocation catalyzed cyclization/cleavage of **8**,[58] viz. hydrogen bonding to phosphate and intracomplex protonation of the leaving methoxide ion (Scheme 23) or deprotonation of the attacking hydroxy group (Scheme 24).

The hydrolysis of nucleoside 2′,3′-cyclic phosphates is also catalyzed by α,ω-alkanediamines. In fact, the catalytic rate constants are greater than with dinucleoside monophosphates.[166,167] The ammonium ion center has been assumed to hydrogen bond to the

Scheme 23



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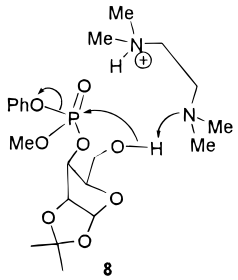
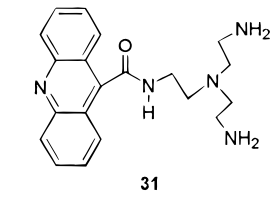
Scheme 24



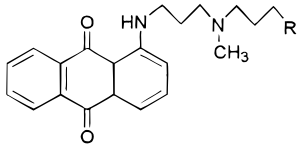
8

phosphodiester monoanion, while the amino group of the same molecule serves as a general base deprotonating the attacking water molecule. The Brønsted β value for the general base catalysis has been determined to be 0.55.

The cleaving activity of diamines toward RNA has been enhanced by tethering the amine to an intercalator.[169] The acridine conjugate **31** cleaved more than 40% of 16S/23S rRNA in 1 h at 37 °C (pH 7.4, [**31**] = 1 mmol L^{-1}). As a first indication of cooperative participation of carboxylate and amino groups in RNA cleavage, the group of Komiyama showed that glycine and iminodiacetic acid conjugates of anthraquinone (**32a**,**b**) cleaved $tRNA^{Phe}$.[170] The carboxylate group was assumed to serve as a general base that deprotonates of the 2′-hydroxy function, and the ammonium ion as a general acid. Interestingly, the cleavage showed some site selectivity: **32a** and **b** cleaved the chain at CpA and GpN sites, respectively.



31



32a: R = -$NHCH_2CO_2H$
32b: R = -$N(CH_2CO_2H)_2$

B. Cleavage by Peptides

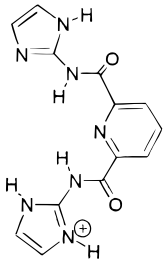
Basic polypeptides containing arginine and/or lysine in addition to hydrophobic amino acid residues accelerate the cleavage of oligo(A) and its dimeric fragment 3′,5′-ApAp.[171−173] Among the peptides studied, poly(Leu-Lys) turned to be the most effective cleaving agent, resulting in an almost 200-fold acceleration in the cleavage of oligo(A) at pH 7.5 when present at 1 mmol L^{-1} concentration in lysine.[173] A β-sheet or α-helical structure of the polypeptide has been suggested to be required for efficient acceleration. According to a tentative model, alignment of the polynucleotide chain between two parallel rows of positively charged amino acid residues results is the origin of the catalytic effect. The hydrophobic amino acid residues facilitate the polypeptide chain

to adopt the required conformation. Besides basic polypeptides, cyclic deca- and tetradecapeptides modestly accelerate the cleavage of oligo(A).[174] In contrast to alkanediamines, the catalytic effect of basic peptides on the hydrolysis of 2′,3′-cyclic phosphates is much weaker than on the cleavage of oligomeric phosphodiesters.[171]

C. Imidazole-Based Cleaving Agents

Bifunctional catalysts bearing two imidazole groups have received considerable interest as cleaving agents, since two histidine residues are known to play a crucial role in the catalytic action of RNase A.[86] Extensive studies have above all been carried out with imidazole-derivatized cyclodextrins, using five-membered cyclic phosphates as substrates.[175] Since these studies have been aimed at elucidating the mechanism of RNase A rather than developing efficient catalysts, their results are discussed separately (see section 7.G).

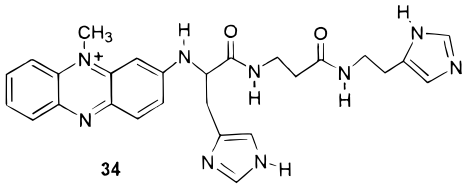
The experimental data on the cleavage of internucleosidic phosphodiester bonds by bis(imidazole) catalysts is scarce. The bis(2-imidazolyl)-substituted pyridine-2,6-carboxamide **33** has been observed to cleave 3′,5′-ApA slowly under neutral conditions (30% cleavage in 4 days at pH 7.5, $T = 50$ °C and [**33**] = 10 mmol L^{-1}).[176] The underlying idea of the design



33

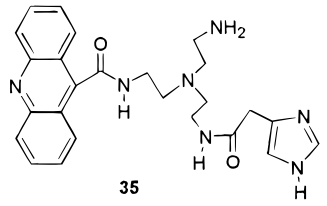
of **33** is that the carboxamido NH groups hydrogen bond to the phosphodiester anion, and one of the imidazole groups deprotonate the attacking 2′-hydroxy function, while the other protonates the leaving 5′-oxygen. Consistent with the assumed bifunctional nature of the catalysis, a bell-shaped pH−rate profile was observed.

More efficient cleaving agents have been obtained by conjugating 4-imidazolyl (and primary amino) groups to an intercalating heteroaromatic moiety.[169,177,178] In all likelihood base-stacking interactions between the intercalating group and the nucleic acid bases results in preequilibrium association of the cleaving agent with RNA, and two nitrogen containing groups then serve as the actual catalysts. The best cleaving agents contain either two imidazole groups, or one primary amino and one imidazole group.[169,177] Naturally the relative positioning of these catalytic groups with respect to the intercalator also affects the cleaving activity. A phenazine conjugate **34** has been reported to cleave 50% of a $tRNA^{Asp}$ transcript in 12 h at pH 7 and 37 °C, when present at 1 mmol L^{-1} concentration.[177] The cleavage rate showed a bell-shaped dependence on pH, refer-



34

ring to a bifunctional mechanism. The chain scission was fastest at the junction of the stem and loop regions. Intercalation may be expected to take place within the double-helical regions, and the cleavage hence occurs at the juxtaposed flexible single-stranded regions.[177] The acridine conjugate **35** has also been shown to exhibit marked catalytic activity. At 1 mmol L^{-1} concentration, this construct cleaved almost 60% of 16S/23S rRNA in 1 h at 37 °C (pH 7.4).[169] The tetrakis(4-imidazolyl) derivative **36** of 3,6-diaminoacridine has been reported to cleave extensively tRNA in 24 h at pH 7 (temperature not indicated) when used at 0.1 mmol L^{-1} concentration.[178] Although the mechanisms of these reactions



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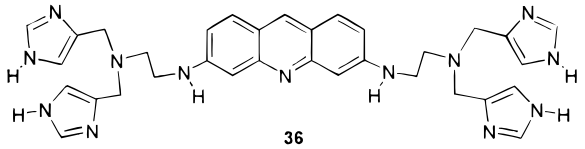
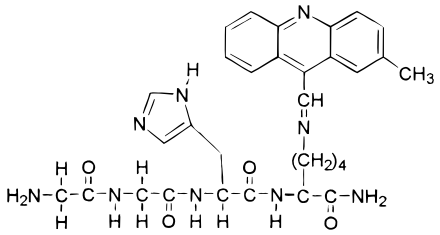


36

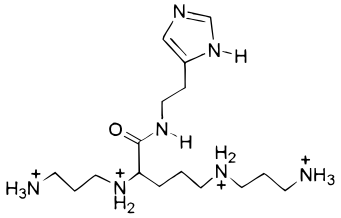
are difficult to deduce with any confidence, it seems clear that cooperativity of two catalytic functions is required for efficient cleavage. Combinations of electrophilic and general base, or general acid/general base catalysis appear as the most attractive alternatives. An ammonium or imidazolium group may serve as an electrophile binding to the anionic phosphodiester group, while the second imidazole group deprotonates the attacking 2′-hydroxy function. Alternatively, the ammonium or imidazolium group may take the role of a general acid and protonate the leaving 5′-oxygen.

Histidine containing tripeptides when tethered to acridine via a lysine-based linker have been shown to cleave ss RNA when the chain is longer than 80 nucleotides.[179] The structural requirement for marked cleaving activity was observed to include a free *N*-terminal primary amino group and histidine (the third amino acid from *N*-terminus) acylated to the α-amino function of the lysine linker. Accordingly, the best cleaving agent was **37**. Owing to the qualitative nature of the analysis by the gel-permeation chromatography, the cleaving effciency could not be quantified. The imidazolyl group of the histidine residue was assumed to deprotonate the 2′-hydroxy function, and the N-terminal α-ammonium group to stabilize the pentacooridinated transition state.



37

Instead of an intercalating group, spermine has been conjugated with an imidazolyl group (**38**) to achieve preequilibrium noncovalent association of the cleaving agent with RNA.[161] This catalyst accelerated the cleavage, but only in imidazole buffers. The imidazolyl group of the cleaving agent was assumed to act as a general base, and the acidic buffer constituent, imidazolium ion, as a general acid. The cleavage pattern obtained closely resembled that described above for **34**.



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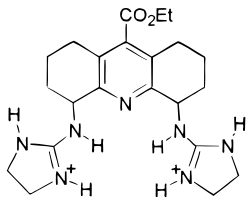
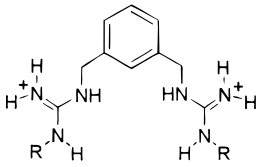
D. Bis(guanidinium)-Based Cleaving Agents

Alcohols substituted with two guanidinium groups constitute another set of compounds that may be regarded as mimetics of nucleases, in this case as models of staphylococcal nuclease.[180] The group of Anslyn has synthesized this type of a receptor by tethering two aminoimidazoline groups to a partially reduced acridine (**39**).[181,182] The receptor binds benzyl phosphate rather tightly even in polar solvents, such as aqueous DMSO ($K = 4 \times 10^3$ L mol^{-1}). Both aminoimidazoline moieties form two hydrogen bonds to the phosphate group. Compound **39** has been shown to cleave mRNA in aqueous solution at micromolar concentrations, but only in the presence of imidazole (0.25 mol L^{-1}).[183] Imidazole is assumed to serve as a general base deprotonating the 2′-hydroxy function of the ribose unit engaged in the substrate/receptor complex. The catalysis is assumed to result from enhanced binding of the transition state to the receptor.



39

The bis(guanidinium) receptor **40a** cleaved 2-hydroxypropyl 4-nitrophenyl phosphate, a mimetic of dinucleoside monophosphate in acetonitrile. The two guanidinium groups have been suggested to bind the phosphodiester group by four hydrogen bonds, and the (dimethyamino)ethyl groups then act as intracomplex general bases deprotonating the attacking hydroxy function.[184] The bound phosphodiester was cleaved 290 times as fast as its free counterpart. Comparable rate acceleration was observed with the core stucture **40b** in the presence of lutidine, but at a higher concentration of the cleaving agent.[185]

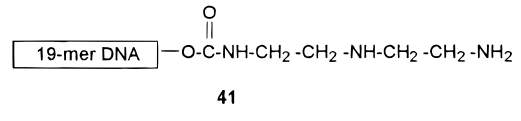


40a: R = $CH_2CH_2N(CH_3)_2$
40b: R = H

When attached to arginine, a bis(guanidinium) catalyst has been shown to cleave TAR−RNA, the transactivation responsive region of HIV-1.[186]

E. Oligonucleotide Conjugates as Sequence-Selective Cleaving Agents

Diethylenetriamine when attached to the 5′-end of a 19-mer DNA oligomer (**41**) has been shown to cleave complementary 30-mer RNA sequence selectively (10% cleavage in 4 h at pH 8, 50 °C).[187] The corresponding triethylenetetraamine conjugate was also active, whereas a 6-aminohexyl tail did not cleave the target chain. When a 19-mer dietylenetriamine conjugate of complementary to the A44-A62 sequence of $tRNA^{Phe}$, a site specific cleavage at the 3′-side of C63 was observed.[188] When the cleaving agent was tethered within a 21-mer oligomer (**42**), a rather slow site specific cleavage of the complementary oligoribonucleotide took place (3% cleavage in 16 h at pH 7.5, 37 °C).[189]



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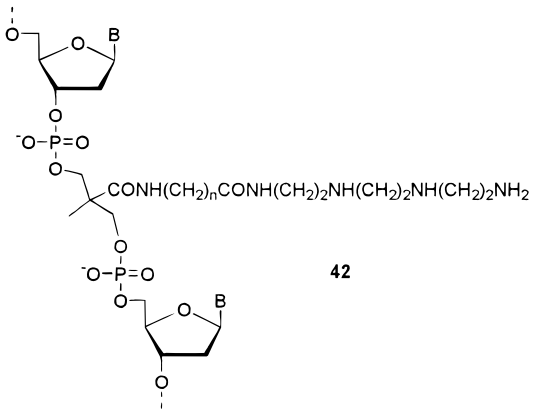
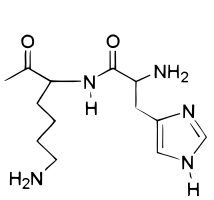


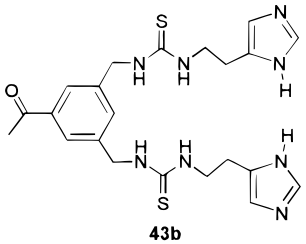
42

The action of bifunctional imidazole-based cleaving agents has been converted sequence selective by attaching them with the aid of a nonnucleosidic building block in the middle of a methylphosphonate oligomer (**43**).[190] These constructs cleaved the RNA target without any additional catalyst, although

rather slowly. Even with the best cleaving agents, the estimated half-life was more than 5 d at 25 °C.



43a



43b

F. Synthetic Polymers as Cleaving Agents

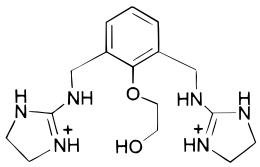
Oligoribonucleotides have been demonstrated to undergo enhanced cleavage at nonterminal PypA and PypC sites (Py stands for a pyrimidine nucleoside) in the presence of polyvinylpyrrolidine.[191] Evidently the amino group of the adenine or cytosine base of the 5′-linked nucleoside participates in the reaction. The stability of these bonds also depends on nearest neighbors. In particular, a purine nucleoside as the 3′-neighbor of a UpA site is rate-accelerating.[191,192]

G. Cleavage of Five-Membered Cyclic Phosphates by RNase Mimetics

Hydrolysis of 4-*tert*-butylcatechol cyclic phosphate, a structural analogue of nucleoside 2′,3′-cyclic phosphates, is one of the most thoroughly studied reactions susceptible to bifunctional catalysis by an enzyme mimetic. β-Cyclodextrins having two of the primary hydroxy functions replaced with imidazolyl groups have been prepared as a model RNase A.[193,194] These bifunctional catalysts hydrolyze 4-*tert*-butylcatechol cyclic phosphate regioselectively to 4-*tert*-butylcatechol 2-phosphate (Scheme 25).[90,193,195] The regioselectivity was, however, observed to be reversed on replacing the imidazolyl groups with (4-imidazolylmethyl)thio groups.[196] The catalytic activity is highest around pH 7, i.e., when the catalyst may be assumed to be partially protonated, and the same catalyst hence contains both an imidazole and imidazolium ion residue. The best catalyst is the one bearing the imidazolyl residues on neighboring sugar units.[194,195] The linear dependence of the square root of the kinetic solvent deuterium isotope effect on the mole fraction of D_2O in the solvent water suggests that two protons are transferred in the rate-limiting step.[197] Mechanistically the reaction has been described as a concerted general acid/base catalysis, where one of the nonbridging phosphate oxygens is protonated by one imidazolium residue, while the other imidazole group deprotonates the attacking water molecule.[90,91,93,175,197] The conclusion that the imidazolium residue protonates a nonbridging oxygen and not the leaving group is based on the fact that the most efficient catalyst was obtained by bonding the two imidazoles to neighboring sugar units of cyclodextrin, not to sugar units on the opposite sides of the cyclodextrin cavity. The latter geometry might be expected to be optimal for the leaving group protonation. If it is assumed that the general base catalyzed attack of the water molecule and general acid-catalyzed departure of the leaving group are concerted, the attacking and leaving oxygens should be 180° degrees apart.[93] In the best catalyst the two imidazole residues are, however, mounted only 51° apart.

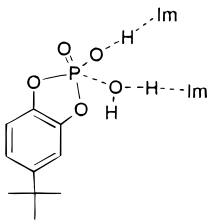
The hydroxide ion catalyzed hydrolysis of nucleoside 2′,3′-cyclic phosphates becomes regioselective in the presence of cyclodextrins. α-Cyclodextrin steers the reaction to 3′-phosphates.[131] It has been suggested that hydrogen bonding of the cyclodextrin hydroxy groups to the O2 of the pyrimidine base and the nonbridging oxygens of the phosphate group brings the O3′ atom into the hydrophobic cavity of the cyclodextrin host, and this results in an unfavorable medium effect on the departure of the 3′-oxygen as alkoxide ion.[131,198] A similar regioselectivity has been achieved with calixarenes.[199] β-Cyclodextrin, in turn, enhances the rupture of the P−O3′ bond.[200] In this case, the regioselectivity has been attributed to inclusion of the nucleic acid base into the cavity, which also forces the O2′ atom into a nonpolar environment. The regioselectivity is enhanced by alkali metal halides.[201] A similar difference in regioselectivity has been observed on cleaving the polymeric cyclic phosphate intermediates formed in RNA hydrolysis in the presence of α- and β-cyclodextrins.[202] Branching of the cyclodextrin with α-D-glucopyranosyl or α-maltosyl groups further improved the catalytic properties.[203]

The intermolecular transesterification of catechol cyclic phosphate with the mimetics of staphylococcal nuclease has been studied rather extensively. The highest rate has been observed with bis(2-aminoimidazoline) derivative **44** that in DMF in the presence of diisopropylethylamine reacts with cyclic catechol phosphate 3.8×10^5 times as effectively as 2-phenylethanol.[204] Monoamidinium derivatives,[205−207] as well as bis(guanidinium) and bis(2-aminoimidazoline) derivatives having either benzene or an acyclic alkane as a spacer moiety,[208] cleaved cyclic catechol phosphate less efficiently.



44

Scheme 25



While the intermolecular transesterifications of cyclic phosphates takes place in organic solvents, it is interesting to note that adenosine 2′,3′-cyclic phosphate has been observed to dimerize in aqueous solution in the presence of poly(U) and oligoamines to a dinucleoside 2′,5′-diphosphate.[162,209] The reaction was suggested to take place within a triple helical

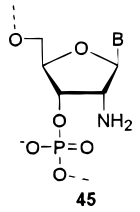
structure formed from two strands of poly(U) and monomeric adenosine 2′,3′-cyclic phosphate.[209] Evidently the 5′-hydroxy function of one nucleotide attacks the cyclic phosphate moiety of another, and this process is somehow facilitated by the amines present in solution.

8. The Effect of Thia and Aza Substitutions on the Transesterification of Ribonucleoside 3′-Phosphodiesters

Hydrolytic reactions of structural analogues of ribonucleoside 3′-phosphoesters having either one of the phosphate oxygens or the attacking 2′-oxygen replaced with sulfur or nitrogen have recently received considerable interest. On one hand, the rapidly expanded use of structurally modified oligonucleotides as tools for molecular biology[33,210,211] has brought the solvolytic stability of such phosphodiester analogues into focus. On the other hand, various thio analogues of oligoribonucleotides have been extensively used as stereochemical probes in mechanistic studies of protein enzymes[212−215] and catalytic ribonucleic acids,[216,217] and the effect of thiosubstitution on kinetics has been exploited in elucidating the rate-limiting step of enzymatic hydrolysis[218,219] and distinguishing between various kinetically equivalent pathways.[220] Accordingly, the importance of proper understanding of the same subtitutional effects on nonenzymatic reactions is obvious.

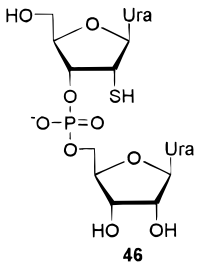
A. Amino and Mercapto Groups as Intramolecular Nucleophiles

Replacing the 2′-hydroxy group of a nucleoside 3′-phosphodiester with an amino function markedly increases the hydrolytic stability. Polynucleotides consisting of 2′-amino-2′-deoxyuridine (**45**; B = uracil) or 2′-amino-2′-deoxycytidine (**45**; B = cytosine) units have been reported to stand treatment with aqueous 0.1 mol L^{-1} potassium hydroxide at 37 °C for 80 min, whereas 90% and 70% of unmodified poly(U) and poly(C) was cleaved.[221] Even the 2′-deoxy-2′-azido analogue of poly(C) was shown to be relatively resistant to the same conditions: 95% of the starting material remained intact.



45

Similarly to the 2′-amino function, the 2′-mercapto function is a much less effective nucleophile than the 2′-hydroxy group toward the neighboring phosphodiester. Neither cleavage nor isomerization of the phosphodiester bond was observed, when 2′-deoxy-2′-thiouridylyl(3′,5′)uridine (**46**) was treated with aqueous 0.1 mol L^{-1} hydrogen chloride for 1 h at 100 °C.[222] The only reaction that took place was dimer-



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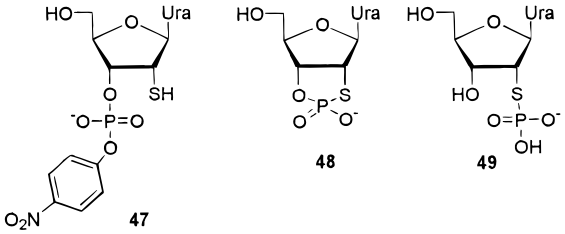
ization of the starting material via a disulfide bond formation. Under alkaline conditions, depyrimidination of the 2′-deoxy-2′-mercaptouridine unit took place faster than any reaction at phosphorus. The 2′-mercapto group has been shown to attack on phosphorus only when the leaving group is strongly electronegative, such as 4-nitrophenyl group in phosphodiester **47**. This compound undergoes transesterification to 2′-*S*,3′-*O*-cyclic phosphorothiolate (**48**) under alkaline conditions, although nucleophilic aromatic substitution at the 4-nitrophenyl moiety takes place concurrently.[223] Rapid subsequent hydrolysis of **48** proceeds exclusively by P−O bond rupture to 2′-*S*-phosphorothiolate (**49**). The pH rate profile of



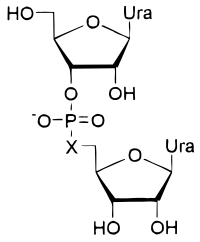
47 **48** **49**

the cleavage of **47** to **48** consists of three regions: the reaction is pH-independent at pH < 6 ($k_{obs} = 10^{-6}$ s^{-1} at 25 °C, $I = 0.2$ mol L^{-1}), first-order in hydroxide ion concentration at pH 6−8, and again pH-independent at pH > 8 ($k_{obs} = 10^{-4}$ s^{-1} at 25 °C, $I = 0.2$ mol L^{-1}). The latter change in the reaction order most likely refers to ionization of the 2′-mercapto group. Accordingly, the 2′-mercapto group is 10^5 times more acidic than the 2′-hydroxy group, whereas the 2′-sulfide anion has been estimated to be 10^7 times less efficient intramolecular nucleophile toward the phosphorus than the 2′-oxyanion.[223]

B. Reactions of 5′-Phosphorothiolates and 5′-Phosphoramidates

The hydroxide ion catalyzed transesterification of uridylyl(3′,5′)5′-deoxy-5′-thiouridine (**50a**) to 2′,3′-cyclic phosphate proceeds 4−5 orders of magnitude faster than the corresponding reaction of 3′,5′-UpU.[224,225] When inserted in RNA, the same thiosubstitution has been estimated to result in up to 10^6-fold rate acceleration.[226] Since thiols are 10^5−10^6 times stronger acids than aliphatic alcohols, a rate acceleration of this magnitude is expected. Under acidic conditions, **50a** is nearly as stable as 3′,5′-UpU,[224,227,228] and isomerization to the 2′,5′-isomer takes place, although more slowly than with 3′,5′-UpU. The existence of isomerization under acidic

conditions reveals that a pentacoordinated thiophosphorane intermediate is formed and may pseudorotate as a neutral species.



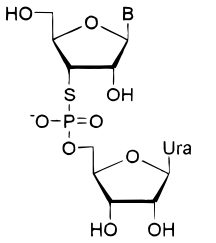
50a: X = S
50b: X = NH

In marked contrast to 3′,5′-UpU and its 5′-thiolate analogue **50a**, the cleavage of uridylyl(3′,5′)5′-deoxy-5′-aminouridine (**50b**) is pH-independent at pH > 6.[225] This pH-independent rate equals to that of the cleavage of 3′,5′-UpU at pH ≈ 13. The cleavage of **50b** is hydronium ion catalyzed at pH 5−6. Accordingly, under neutral and mildly acidic conditions **50b** is several orders of magnitude less stable than 3′,5′-UpU, and reacts at neutral pH as rapidly as the 5′-thiolate **50a**. The pH independence of the cleavage of **50b** at pH > 6 has been interpreted to indicate that the P−N bond is cleaved by kinetically visible protonation of the nitrogen atom, i.e., the nitrogen atom may depart only as an amine, not as an amide ion.[225] While increasing alkalinity enhances deprotonation of the attacking nucleophile, the 2′-hydroxy group, it simultaneously retards protonation of the leaving group. The hydronium ion catalyzed reaction may analogously been attributed to the attack of 2′-hydroxy group on protonated phosphoramidate bond.

C. Reactions of 3′-Phosphorothiolates and 3′-Phosphoramidates

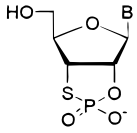
The internucleosidic 3′-*S*,5′-*O*-phosphorothiolate linkage also is cleaved in aqueous alkali considerably more readily than the phosphodiester bond. The values reported for this "3′-bridging thio-effect" of hydroxide ion catalyzed cleavage of 3′,5′-UpU and its 3′-thiolate analogue **51** range from 200 [227] to 2000.[228] The rate-enhancing effect is thus smaller than that of 5′-thiosubstitution, which is expected since the leaving group is now an alkoxide ion, not a sulfide ion. According to Weinstein et al.,[228] the 3′-thiosubstitution does not markedly affect the pK_a of the 2′-hydroxyl function. Accordingly, nucleophilic attack of the 2′-oxyanion on the 3′-*S*-phosphorothiolate monoanion appears to be greatly enhanced compared to the attack on phosphodiester monoanion. Under acidic conditions, the thio effect is close to unity. Possibly more difficult protonation of the 3′-phosphorothiolate bond compared to the phosphodiester bond compensates the intrinsically more facile attack of the 2′-hydroxy group on phosphorothiolate group.

Cleavage of the 3′-*S*-phosphorothiolate linkage yields a 2′-*O*,3′-*S*-cyclic phosphorothiolate (**52**). The subsequent hydrolysis of this intermediate takes place under alkaline conditions by direct displacement of the 2′-alkoxy ligand by hydroxide ion.[227,228] In acidic solutions a thiophosphorane intermediate



51a: B = Uracil
51b: B = Hypoxanthine

is obtained and it may pseudorotate. The intermediate then degrades entirely via release of the thiolate ligand.[228] These observations and conclusions appear to be consistent with those described earlier for hydrolysis of *O*,*S*-ethylene phosphorothiolate.[229,230]



52

D. Reactions of Phosphorothioates and Phosphoramidates Bearing Substitutions in Nonbridging Position

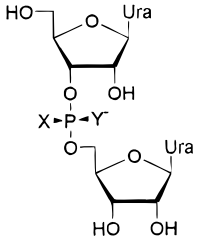
Substituting sulfur for either of the nonbridging oxygens has practically no effect on the cleavage rate in aqueous alkali, where intramolecular transesterification of RNA phosphodiester linkages to 2′,3′-cyclic phosphodiesters is suggested to proceed by an associative "in-line" mechanism via a dianionic pentacoordinated a transition state (Scheme 1). All the values reported for the thio effect (k_{P-O}/k_{P-S}) of this reaction are close to unity (Table 6), the S_P diastereomers (**53a**) being slightly more reactive than their R_P counterparts (**53b**). No desulfurization occurs, indicating that the alkoxy group rather than the sulfur ligand adopts an apical position in the trigonal-bipyramidal transition state.[237] The hydroxide ion catalyzed cleavage hence entirely leads to a stereospecific formation of a 2′,3′-cyclic monophospho-

Table 6. Kinetic Thio Effects in Hydroxide Ion Catalyzed Cleavage of Ribonucleotide Phosphodiesters: Thisubstitution of a Nonbridging Oxygen

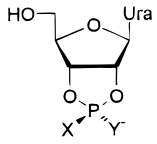
compd	solution	T, °C	k_{P-O}/k_{P-S}	ref
(R_P)-Up(s)A[a]	0.5 mol L^{-1} KOH	37	1.3	231
(S_P)-Up(s)A[b]	0.5 mol L^{-1} KOH	37	1.06	231
(R_P)-Up(s)U, **53b**	pH 9−12	50	1.3	232
(S_P)-Up(s)U, **53a**	pH 9−12	50	0.78	232
(S_P)-pGp(s)AGU	0.2−0.5 mol L^{-1} NaOH	37	1.1	219
(R_P)-3′-UMPS-OAr[c]	pH 8.5−11	25	1.7−1.8	233
(S_P)-3′-UMPS-OAr[c]	pH 8.5−11	25	2.4−2.5	233
(R_P)-2′,3′-cUMPS, **54b**	0.25 mol L^{-1} KOH	37	6	234
(R_P)-2′,3′-cUMPS, **54b**	pH 10−12	90	2.1	235
(S_P)-2′,3′-cUMPS, **54a**	pH 10−12	90	1.4	235

[a] The analogue of **53b** having the 5′-linked uridine replaced with adenosine. [b] The analogue of **53a** having the 5′-linked uridine replaced with adenosine. [c] Several *O*-aryl esters were studied, pK_a(ArOH) varying from 9.95 to 7.14. The compounds are analogues of **2** having one of the nonbridging oxygens replaced with sulfur (B = uracil).

rothioate (**54a**,**b**). The reaction proceeds by complete inversion at phosphorus.[237] A similar observation has recently been reported for hydrolysis of the monothioate analogues of an RNA oligomer [5′-UCGUp(s)AA-3′] in the presence of polyvinylpyrrolidine.[238]

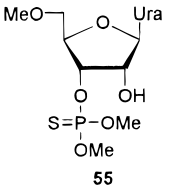


53a: X = S; Y = O
53b: X = O; Y = S



54a: X = S; Y = O
54b: X = O; Y = S

Under neutral and acidic conditions the transesterification takes place via a phosphorane intermediate, instead of a pentacoordinated transition state. The thiophosphorane intermediate is sufficiently long-lived to pseudorotate, as evidenced by the fact that interconversion of **53a** and **53b** competes with the transesterification to **54a**,**b** and desulfurization to 2′,5′- and 3′,5′-UpU.[237] Migration of the thiophosphate was shown to be stereospecific, as expected, proceeding with retention of configuration at phosphorus.[237] The fact that desulfurization effectively competes with rupture of the P−OR bonds seriously complicates the interpretation of experimental results, since now two different thio effects must be distinguished: the effect of thiosubstitution on (i) the overall hydrolytic stability (transesterifications + desulfurization) and (ii) the rate of transesterifications (isomerization and cleavage). The magnitude of both of these thio effects depends on the ionic form of the pentacoordinated intermediate, through which the reaction takes place. The overall thio effect, k_{P-O}/k_{P-S}, on pH-independent reactions of monoanionic phosphodiesters (cf., Scheme 9) is 0.56 and 1.2 with R_P- (**53b**) and S_P-isomer (**53a**), respectively.[237] The predominant reaction is, however, desulfurization, especially with the more reactive R_P-diastereomer. The thio effects on the cleavage to **54a**,**b** are 0.09 (for R_P) and 0.3 (for S_P), and those on the transesterification to the 2′,5′-isomer 5.1 (R_P) and 6.9 (S_P).[237] Accordingly, the thiosubstitution appears to accelerate the departure of the 5′-linked nucleoside from the pentacoordinated intermediate, but retard the isomerization via pseudorotation. On going to solutions where the transesterifications take place via a neutral phosphorane (Schemes 4 and 7) the thioate analogues become hydrolytically more stable than 3′,5′-UpU, the reactivity difference being continuously increased with the decreasing pH. The pH−rate profiles clearly show that while 3′,5′-UpU reacts at pH < 2 via a monocationic phosphorane intermediate (Scheme 4), **53a** and **53b** utilize under the same conditions a route via a neutral pentacoordinated intermediate (cf., Scheme 7).[237] Accordingly, the observed overall thio effect depends on pH, being about 2 at pH 2 and up to 100 in aqueous 1 mol L^{-1} hydrogen chloride.[237] The thio effect on the isomerization of **56a**,**b** to the corresponding 2′,5′-isomers via a neutral pentacoordinated intermediate is about 10. The effect on the cleavage to **54a**,**b** cannot be accurately estimated, owing to fast subsequent decomposition of the latter.



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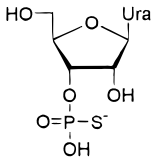
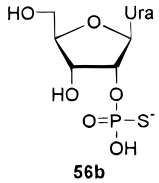


56a



56b

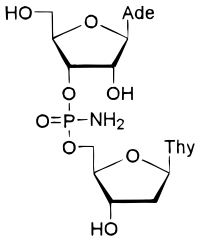
An upper limit for the thio effects on hydronium ion catalyzed reactions of 3′,5′-UpU has been estimated by comparing the reaction rates of uridine 3′-(dimethyl phosphate) (**5b**) with those of uridine 3′-(dimethyl phosphorothioate) (**55**).[47] Thiosubstitution retards the hydronium ion catalyzed isomerization and cleavage by a factor of 50 and 300, respectively. The major factor behind these large effects in all likelihood is more difficult protonation of the thio analogue, although this effect has not been quantitatively distinguished from the altered susceptibility to nucleophilic attack.[47]

The effect of thiosubstitution on hydrolysis of nucleoside 2′,3′-cyclic phosphodiesters (**4**) closely resembles that on the transesterification of 3′,5′-UpU discussed above. In aqueous alkali, the hydrolysis proceeds via a dianionic pentacoordinated transition state obtained by the attack of hydroxide ion on the tetracoordinated phosphorus of **4** or **54a**,**b**. The thio effect on this reaction is rather modest (Table 6).[235] Desulfurization does not compete with the hydrolysis, but only a mixture of nucleoside 2′- and 3′-thiomonophosphates accumulates.[234,235] Under neutral and acidic conditions, a pentacoordinated intermediate is obtained by an attack of water on anionic, neutral, or cationic cyclic phosphodiester (Scheme 20). Again this intermediate, when derived from monothioates (**54a**,**b**), undergoes desulfurization in addition to hydrolysis. The observed overall thio effect is pH dependent, analogous to transesterification of **53a**,**b**. The thio effects on the hydrolysis via a monoanionic phosphorane are 3.4 (for R_P) and 1.7 (for S_P), and those on the hydrolysis via a neutral phosphorane 16 (for R_P) and 11 (for S_P).[235]

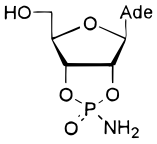
Nucleoside 2′- and 3′-phosphates undergo hydrolytic dephosphorylation by a dissociative mechanism (Scheme 21). The effect of thiosubstitution on this reaction is rate accelerating: nucleoside 3′- and 2′-phosphoromonothioates (**56a**,**b**) are hydrolyzed 300 and 260 times as fast as their oxygen counterparts.[236] Evidently the large rate-accelerating thio effect reflects the easy formation of the relatively stable thiometaphosphate intermediate.[147]

Quantitative data on the transesterification of dinucleoside 3′,5′-phosphoramidates is meager. Com-

pound **57** has been shown to decompose readily in neutral aqueous solution to a complicated product mixture, from which the cyclic phosphoramidate **58** could be identified.[239,240]



57



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