Review

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Structural modifications as tools in mechanistic studies of the cleavage of RNA phosphodiester linkages

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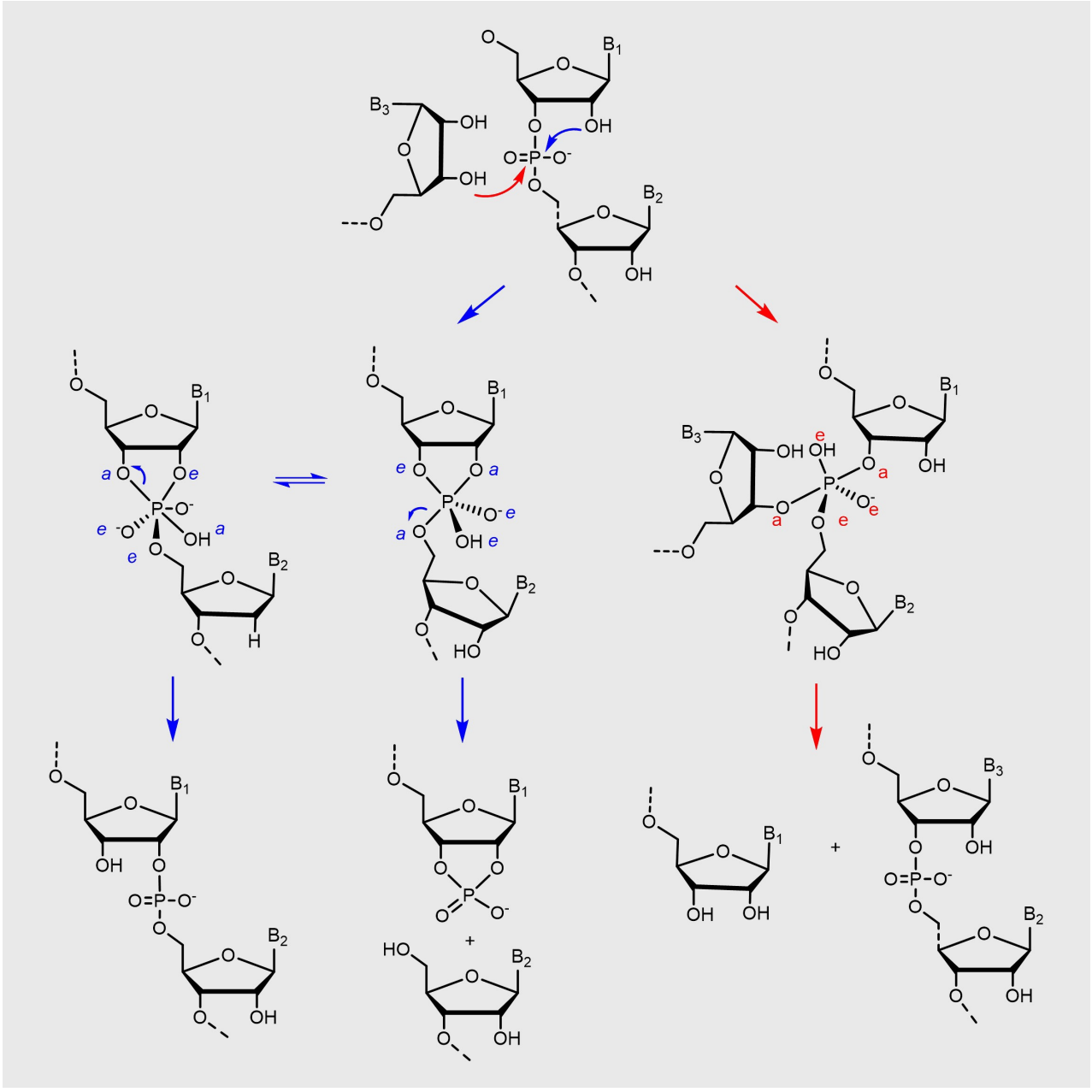

Abstract: The cleavage of RNA phosphodiester bonds by RNase A and hammerhead ribozyme at neutral pH fundamentally differs from the spontaneous reactions of these bonds under the same conditions. While the predominant spontaneous reaction is isomerization of the 3',5'-phosphodiester linkages to their 2',5'-counterparts, this reaction has never been reported to compete with the enzymatic cleavage reaction, not even as a minor side reaction. Comparative kinetic measurements with structurally modified di-nucleoside monophosphates and oligomeric phosphodiesters have played an important role in clarification of mechanistic details of the buffer-independent and buffer-catalyzed reactions. More recently, heavy atom isotope effects and theoretical calculations have refined the picture. The primary aim of all these studies has been to form a solid basis for mechanistic analyses of the action of more complicated catalytic machineries. In other words, to contribute to conception of a plausible unified picture of RNA cleavage by biocatalysts, such as RNAse A, hammerhead ribozyme and DNAzymes. In addition, structurally modified trinucleoside monophosphates as transition state models for Group I and II introns have clarified some features of the action of large ribozymes.

Keywords: RNA, mechanism, pentaoxyphosphorane, phosphodiester cleavage, phosphodiester isomerization

1. Introduction

The cleavage of RNA phosphodiester linkages by ribonucleases, catalytic ribonucleic acids (ribozymes) and catalytic deoxyribonucleic acids (DNAzymes) is a subject of continued interest, largely owing to the increasing role of RNA as drug targets, drugs and vaccines.[1,2] Ribonucleases and the so-called small ribozymes (50–150 nucleotides long catalytic sequences) utilize a common overall pathway: the 2'-O attacks on the vicinal phosphate group and displaces the 5'-O with concomitant formation of a 2',3'-cyclic phosphodiester. Hydrolysis of the latter to a mixture of 2'- and 3'-phosphomonoesters then completes the reaction (Scheme 1).[3] With introns I and II (large self-splicing sequences of hundreds of nucleotides), the reaction is intermolecular. The attacking nucleophile is either the 2'-O or 3'-O of an external nucleoside (or a water molecule) and the departing nucleophile 3'-O.[4] DNAzymes utilize an intramolecular mechanism, at least in most thoroughly studied cases: 2'-O attacks and 5'-O departs.[5]

Both an intra- or inter-molecular attack on the scissile phosphodiester linkage gives a pentaoxyphosphorane intermediate (or transition state)[6] that breaks down by departure of either 3'-O or 5'-O of the phosphodiester attacked. Which one serves as a leaving group depends on interaction with external species, such as specific and general acids and bases, H-bond donors or metal ions.

Although RNA is hydrolytically much less stable than DNA, the un-catalyzed cleavage of an individual phosphodiester linkage still is very slow, the half-life under physiological conditions (pH 6–7, 25 °C) being of the order of 10 years.[7] RNase A, the most thoroughly studied metal-ion-independent ribonuclease,[8] results in a 10^{11}-fold rate acceleration when bound to a 5'-UpA-3' site of RNA,[9] although the catalytic machinery appears quite simple: two histidine residues are directly involved in the catalytic event. The most efficient small ribozyme, Hammerhead ribozyme, having only nucleobases as catalytic entities, is 2–3 orders of magnitude less efficient.[10] Mg^{2+} ion, however, may participate in the latter case.[11] What is the source of these enormous rate-accelerations?

For both RNase A and Hammerhead ribozyme, three different mechanisms appear feasible *a priori*. Firstly, general base catalyzed attack of the 2'-OH may give a highly unstable di-anionic phosphorane intermediate that breaks down to a 2',3'-cyclic phosphate and free 5'-linked nucleoside without any additional catalysis (A in Scheme 2). Secondly, general base catalyzed deprotonation of the 2'-OH and general acid

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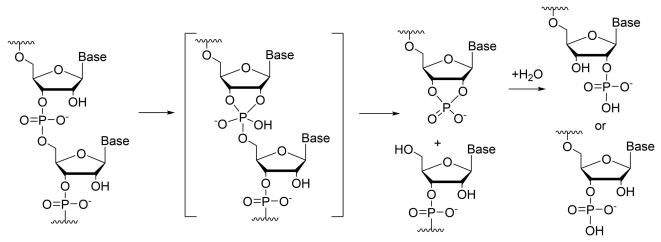
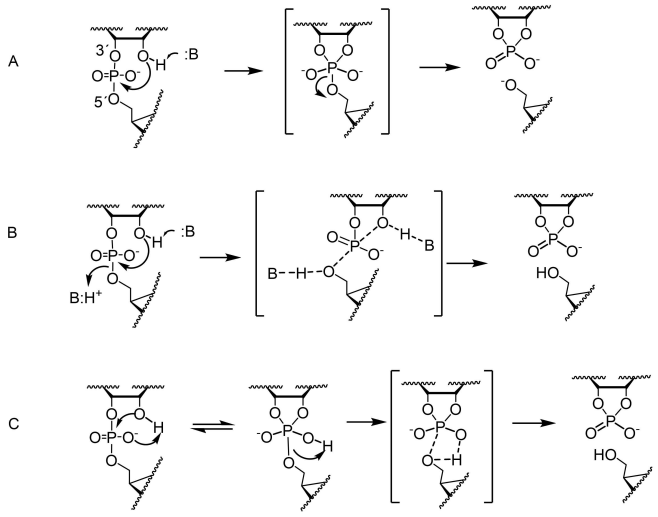


Scheme 1. Cleavage of RNA phosphodiester bonds by ribonucleases and small ribozymes.



Scheme 2. Alternative mechanisms for the cleavage of RNA phosphodiester linkages by ribonucleases and small ribozymes.

catalyzed protonation of 5'-O could take place in a concerted manner via a single transition state (B in Scheme 2). Thirdly, the attack of 2'-OH might take place concerted with intramolecular proton shuttling to a non-bridging phosphoryl oxygen. In this case, a mono-anionic phosphorane intermediate obtained has a finite lifetime, and subsequent intramolecular proton shuttling from the intermediate to the departing 5'-O then results in the bond cleavage (C in Scheme 2).

Majority of the mechanistic studies with RNase A have lent support for a B type mechanism: two His residues participate, one provides an imidazole base that deprotonates the attacking 2'-OH, and the other one an imidazolium ion moiety that protonates the departing 5'-O.[12–17] Nevertheless, mechanism C has also received some support.[18–20]

With small ribozymes, the proton transfer machinery is more complicated, consisting of only nucleobases and hydrated metal ions in addition to the attacking 2-hydroxyl group. The underlying principle of catalysis still is the same: transfer of proton form the attacking 2'-OH to the departing 5'-O. Excellent recent reviews on the catalytic mechanisms of small ribozymes[21–23] and insightful papers on the mechanism of 8–17 DNAzyme[24,25] are available.

With hammerhead ribozyme, for example, the proton transfer is based on two guanosine nucleosides.[26] To generate a general acid – general base system, one guanosine (G12) must donate the N1H proton to another one (G8). According to X-ray structure, a water-mediated proton transfer to the 2'-OH of G8 takes place. As a result, deprotonated N1 of G12 serves as a general base and protonated 2'-OH of G8 as a general acid.[27] However, as mentioned above, two Mg^{2+} ions may be involved. One coordinated to O^6 of G12[28] facilitates deprotonation of N1H, and the other one provides a water molecule that possibly takes the role of general acid.[29] According to an alternative widely accepted mechanism, a Mg^{2+} ion that bridges the scissile phosphate (C17) to another phosphodiester bond (A9) simultaneously interacts with 2'-OH of G8 increasing its acidity and, hence, ability to serve as a general acid.[30]

In the absence of biocatalysts, RNA phosphodiester linkages react along a different pathway. The predominant uncatalyzed reaction under neutral conditions is isomerization to a 2',5'-diester instead of transesterification to a 2',3'-cyclic phosphate with concomitant departure of the 5'-linked nucleoside.[31] It is worth noting that isomerization is the predominant reaction also with polyuridylic acid,[32] not only with dinucleoside-3',5'-monophosphates used extensively as minimalistic models in mechanistic studies. Comparative kinetic measurements with structurally modified di-nucleoside monophosphates and oligomeric phosphodiesters have played an important role in clarification of mechanistic details of the buffer-independent and buffer-catalyzed reactions. The primary aim has been to form a solid basis for mechanistic analyses of the action of more complicated catalytic machineries. In other words, to contribute to conception of a plausible unified picture of RNA cleavage by metal-ion-independent biocatalysts, such as RNAse A and hammerhead

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ribozyme. The conclusions achieved by these studies form the first major topic of the present review.

At high concentrations of imidazole buffer, cleavage of phosphodiester bonds becomes faster than isomerization, but both reactions still compete.[33,34] The second topic discussed consists of various mechanistic interpretations of general acid/base catalyzed cleavage and isomerization.

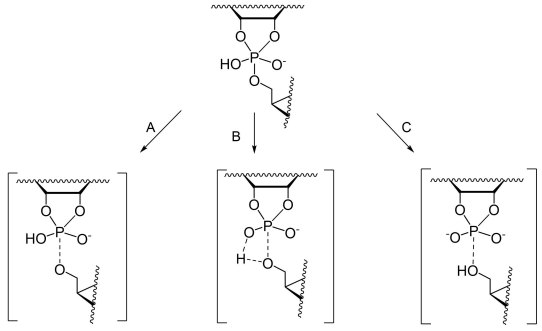
Oligonucleotides having one or two of the phosphoryl oxygen atoms in a phosphodiester linkage replaced with sulfur, nitrogen or carbon are extensively used in mechanistic enzymology and development of oligonucleotide based drugs. The mechanistic consequences that such structural modifications have on the kinetics and mechanisms of phosphodiester cleavage form the third subject briefly discussed.

The fourth topic concerns the role of structurally modified nucleotides in construction of transition state models for the action of large ribozymes. With large ribozymes, the attacking nucleophile is not the 2'-OH adjacent to the scissile phosphodiester linkage, but a hydroxyl function of an external nucleoside.[4] Mechanistic studies with simplified mimics of the phosphorane intermediate/transition state bearing three different nucleosides have shed light to some mechanistic details of the catalytic action of large ribozymes.

2. Buffer-independent reactions of RNA phosphodiester linkages

2.1. Studies with small molecular models

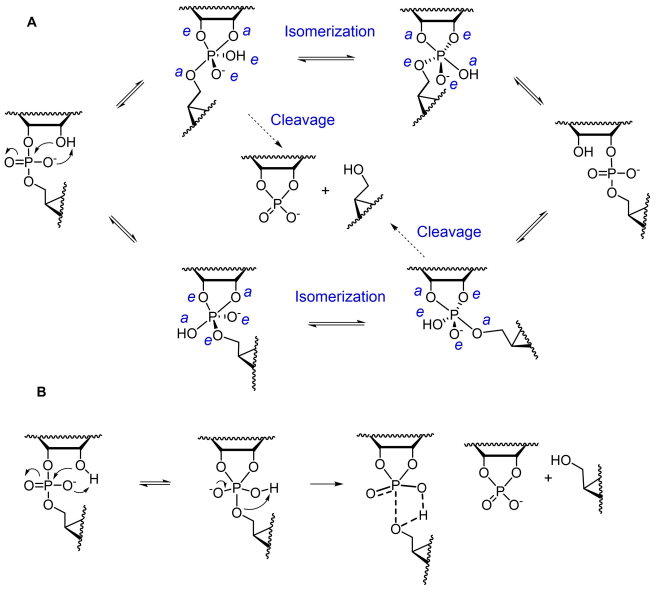
Kinetic studies with dinucleoside-3',5'-monophosphates over a wide pH-range (0–12 at 90 °C) have shown that the predominant buffer-independent reaction of RNA 3',5'-phosphodiester linkages under neutral conditions is pH-independent isomerization to 2',5'-linkages.[31] This reaction is with uridylyl-3',5'-uridine (3',5'-UpU) 46 times as fast as the pH-independent cleavage by departure of the 5'-linked uridine. Since both cleavage and isomerization are pH-independent, they take place via the predominant ionic form, *i. e.* by un-catalyzed conversion of the phosphodiester mono-anion to a mono-anionic phosphorane, followed by un-catalyzed breakdown of the phosphorane mono-anion to the isomerization and cleavage products. As regards the cleavage, breakdown of the intermediate must be the rate-limiting step since isomerization is much faster than cleavage. Three alternatives exist for timing of the proton transfer from the phosphorane to the departing nucleoside. Fission of the P–O5' bond may precede proton transfer to O5' (A in Scheme 3), P–O5' bond may be cleaved concerted with proton shuttling (B in Scheme 3), or proton transfer to O5' precedes bond fission (C in Scheme 3). As discussed below in more detail, alternative B appears most attractive. Proton transfer concerted with bond cleavage stabilizes both products, 2',3'-cyclic



Scheme 3. Alternatives for the timing of the proton transfer from the phosphorene intermediate to the departing nucleoside.

phosphate mono-anion and neutral nucleoside. Formation of the phosphorane mono-anion may well take place by a similar proton shuttling mechanism. Transfer of proton from the attacking 2'-OH to a phosphoryl oxy-ligand simultaneously increases the nucleophilicity of 2'-O and electrophilicity of the phosphorus atom.

The phoshorane intermediate formed by one of the mechanisms in Scheme 3 is a trigonal bipyramid with two apical and three equatorial oxygen atoms (Scheme 4).[6] Nucleophiles may enter and depart via apical position only. When the ligand is present as oxyanion, it tends to be equatorial. Upon formation of a five-membered ring, one of the oxygen atoms must be apical and the other equatorial. According to these Westheimer's rules, 2'-O is apical and 3'-O



Scheme 4. pH- and buffer-independent isomerization (A) and cleavage (B) of RNA phosphodiester linkages.

equatorial within the initially formed phosphorane. Either 5'-O or P–OH is apical, the other one being equatorial. P–O$^-$ remains equatorial. In case 5'-O is apical, it may depart. As mentioned above, the proton transfer from P–OH to 5'-O may in principle take place either prior to, concerted with or after the fission of the PO-bond (Scheme 3). Isomerization, in turn, is possible only after a conformational rearrangement of the phosphorane intermediate, the so-called Berry pseudorotation: apical ligands take an equatorial position and two of the equatorial ligands an apical position.[35] Configuration around phosphorus remains retained.[36,37] Since P–O$^-$ tends to remain equatorial, 3'-O inevitably becomes apical and, hence, able to depart. A prerequisite for isomerization, hence, is a phosphorane intermediate that is sufficiently stable to pseudorotate.

Comparison of conventional structure-reactivity correlations has offered a simple approach for distinguishing between the mechanisms in Schemes 3 and 4. The 5'-linked nucleoside in 3',5'-UpU was replaced with an aliphatic alcohol, the acidity of which was systematically varied, and the β_{lg} value was determined for hydronium ion catalyzed, pH-independent, and hydroxide ion catalyzed cleavage (β_{lg} is the slope of log k *vs.* pK_a of the departing alcohol). The β_{lg} value for the hydronium ion catalyzed cleavage that proceeds by pre-equilibrium protonation of the phosphate group and subsequent departure of the leaving group as alcohol, is -0.12 ± 0.05.[38] The hydroxide ion catalyzed reaction that proceeds by departure of alkoxide ion, as discussed below in more detail, is much more sensitive to electron withdrawal by the leaving group: $\beta_{lg} = -1.28 \pm 0.05$.[38] The β_{lg} value, -0.59 ± 0.12, obtained for the pH-independent cleavage[39] falls midway between the values of hydronium and hydroxide ion catalyzed reactions, which represent early and late transition state for the PO-bond cleavage, respectively. In other words, the β_{lg} value is consistent with a transition state where both the proton transfer to the leaving oxygen and the P–O5' bond cleavage are half advanced (B in Scheme 4).

Studies with uridine 3'-dialkylphosphates lend some support for the suggested proton shuttling in the rate-limiting step. The mono-anionic phosphorane obtained as an intermediate of the hydroxide ion catalyzed cleavage of symmetric 3'-dialkylphosphates of uridine (A in Figure 1) serves as a model for the phosphorane intermediate of pH-independent cleavage of their 3'-monoalkyl counterparts (B in Figure 1). With phosphorane A, the oxyanion ligand remains locked to an equatorial position, but the presence of two alkoxy ligands still allows Berry permutation and, hence, both isomerization and cleavage are possible. In fact, the partition of phosphorane A largely favors isomerization, much more than the partition of phosphorane B. The ratio k_{is}/k_{clv} is of the order of 10^5 with phosphorane A, while it ranges from 10 to 100 with phosphorane B (k_{is} and k_{clv} are rate constants for the isomerization and cleavage reactions, respectively).[40] The marked

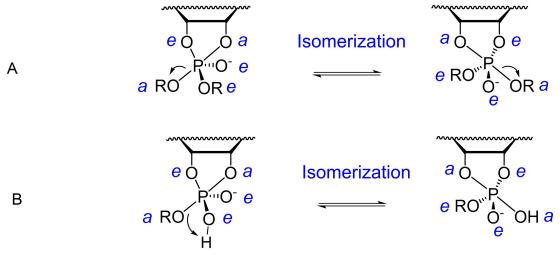


Figure 1. The mono-anionic phosphorane derived from symmetric 3'-dialkylphosphates of uridine (A) as a model for the phosphorane intermediate of pH-independent cleavage of their 3'-monoalkyl counterparts (B).

difference in product distribution most likely originates from the fact that phosphorane A does not contain a rapidly exchangeable proton that could undergo an intramolecular transfer to the leaving group. In other words, intramolecular proton shuttling seems to offer for the rate-limiting PO-bond cleavage of phosphorane B a pathway that is more favorable than that available for phosphorane A.

Density-functional theory (DFT) calculations in gas phase together with estimation of solvation effects by the polarizable continuum model (PCM) have lent support for proton shuttling as an essential feature of the rate-limiting PO-bond cleavage.[41] A couple of questions concerning mechanism B (Scheme 3) still need attention. The pK_a values estimated by various experimental[42] and computational[43] methods for dissociation of an equatorial hydroxyl ligand of phosphoranes range from 8 to 10, while dissociation of an apical ligand is even less favored. Accordingly, one may ask whether the phosphorane really pseudorotates as a monoanion, or does the process take place via kinetically invisible protonation and subsequent deprotonation. DFT calculations with various solvation models, including polarizable continuum model (PCM), continuum COnductor-like screening MOdel (COSMO) and SM5.42R solvation model (SM5), suggest that a mono-anionic phosphorane has a finite life-time and the barrier for its pseudorotation is even lower than that for its neutral counterpart.[44] Consistent with pre-equilibrium isomerization and rate-limiting P–O5' bond fission, the barrier for an endo-cyclic bond cleavage is much lower than that for the exocyclic cleavage. For the phosphorane derived from methyl ethylene phosphate, the barriers are 3.2 and 14.4 kcal mol^{-1}, respectively.[45] Assuming that pseudorotation is a rapid pre-equilibrium, the distribution to isomerization and cleavage products is independent of the equilibrium between various conformers, according to Curtin-Hammett principle.

Another mechanistically interesting question is whether the intramolecular proton transfer from the 2'-OH to non-bridging phosphoryl oxygen and further to departing 3'- or 5'-oxygen takes place as indicated in Scheme 3, or is it mediated

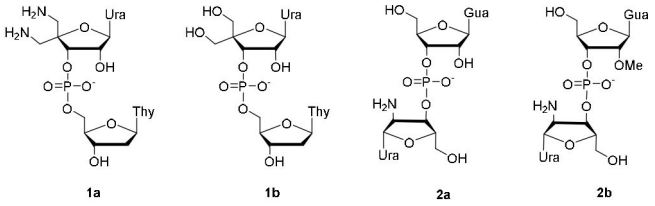
by a water molecule. Uridine 3'-(3-nitrobenzyl)phosphate does not undergo isomerization in *tert*-butanol and is hydrolyzed only reluctantly, evidently by a hydroxide-ion-catalyzed mechanism that does not involve proton-shuttling.[46] This might be an indication of active role of water in proton transfer. More recent combined quantum chemical and molecular mechanical calculations (QW/MM) do not lend support, however, for acceleration of isomerization by participation of water, although they do not strictly exclude it, either.[47]

Besides pH-independent cleavage, hydroxide-ion-catalyzed cleavage is of interest, owing to its repeated usage as a reference reaction for biocatalysis. This reaction is at pH 6 (90 °C) one order of magnitude slower than the pH-independent isomerization and, hence, only 50 % slower than the pH-independent cleavage.[31] In striking contrast to the pH-independent reaction, no isomerization takes place. Attack of 2'-oxyanion on phosphorus evidently gives a di-anionic phosphorane intermediate that is not sufficiently stable to undergo pseudorotation but collapses rapidly to cleavage products. The two negatively charged non-bridging oxygen atoms prefer equatorial position preventing pseudorotation. DFT calculations have given a value of 30 kcal mol^{-1} for the pseudorotation barrier.[48] The transition state is late, the formation of the P–O2' bond and the cleavage of the P–O5' bond being both far advanced.[38] The phosphorane species formed, however, still is an intermediate rather than a transition state. Evidence for this claim comes from the shape of the log k vs. pK_a^{lg} plot over a wide pK_a range covering both alkyl (pK_a 12.5–17.5)[38] and aryl (pK_a 5–10)[49] leaving groups. At pK_a^{lg} = 12.6, a convex break occurs and the slope referring to 3'-alkylphosphates (-1.28) turns to a value of 0.52.[50] The latter value is typical for 3'-arylphosphates that have an early transition state with modestly advanced P–O5' bond cleavage.[51] According to DFT calculations, the 2,2,2-trichloroethyl ester falling at the break point really is a compound with which the barriers for the formation and breakdown of the phosphorane intermediate are roughly equal.[52]

As mentioned above, the transition state of the hydroxide-ion-catalyzed cleavage is late. The β_{eq} value, *i. e.* the equilibrium constant for nucleophilic displacement of aryl-phosphate mono-anions, has estimated to be −1.7.[53] Assuming that this value is also valid for the attack of 2'-O$^-$ on internucleosidic phosphodiester mono-anion, β_{lg} value −1.28 means that the P–O5' bond is 70 % cleaved at the transition state. The elegant studies of the group of Piccirilli, in turn, have shown that the P–O2' bond formation has proceeded rather far in the transition state. A number of C2'-fluoromethyl uridines (CH_3, CH_2F, CHF_2, CF_3) were incorporated into an oligodeoxyribonucleotide and cleavage rate of the scissile 5'-UpN-3' bond (here U refers to C2'-fluoromethyl uridine and N to a 2'-deoxynucleoside) was measured under alkaline conditions as a function of pH.[54] The pH value where the first-order dependence on hydroxide ion concentration turned to zero-order dependence was the pK_a of the attacking 2'-OH. The plot log k vs. pK_a then gave the β_{nuc} value 0.75 ± 0.15 (β_{nuc} is the slope of log k vs. pK_a of the attacking nucleophile), which was large enough to conclude that the P–O2' bond was roughly 50 % formed in the transition state.[54]

Consistent with the conclusions based on structure reactivity correlations, the primary kinetic ^{18}O isotope effect obtained with 5'-UpG-3' for the attacking 2'-O is inverted, $^{16}k_{nuc}/^{18}k_{nuc} = 0.984 \pm 0.004$ ($^{16}k_{nuc}$ and $^{18}k_{nuc}$ are the rate constants for the attack of 2'-^{16}O and 2'-^{18}O, respectively), and that for the leaving 5'-O is normal, $^{16}k_{lg}/^{18}k_{lg} = 1.034 \pm 0.004$ ($^{16}k_{lg}$ and $^{18}k_{lg}$ are the rate constants for the departure of 5'-^{16}O and 5'-^{18}O, respectively).[55] The latter value, for example, is much larger than the one, $^{16}k_{lg}/^{18}k_{lg} = 1.0059 \pm 0.0004$, reported for the cleavage of uridine 3'-(4-nitrophenylphosphate).[56] When the leaving group is as good as 4-nitrophenol, the reaction is a concerted single step process, the departure of the leaving group being only modestly advanced in the transition state.[50]

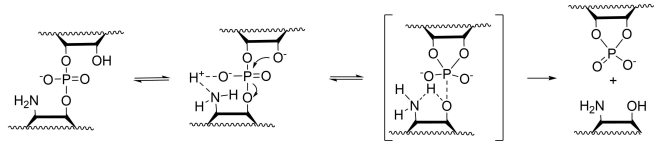
H-bond donors and acceptors close to the reaction center may well affect the course of enzymatic cleavage of RNA phosphodiester linkages, not only by actual H-bond formation but also by electrostatic effects.[57] A well-known example is the terminal ammonium group of Lys41 of RNase A that most likely stabilizes the phosphorane-like transition state.[12] A structural analog of 3',5'-UpT, *viz.* 4'-aminomethyl-5'-amino-5'-deoxyuridylyl-3',5'-thymidine (**1 a**), has been used as a chemical model to learn more about this subject.[58] While the corresponding dihydroxy analog (**1 b**) reacts over the entire pH range like 3',5'-UpU, **1 a** behaves very differently in the pH range 3–9. The amino functions of **1 a** are protonated sequentially, the first one at pH 7.2 and the second at pH 5.8. At pH 3–5, *i. e.* when both of the amino groups were protonated, isomerization and cleavage were pH-independent. Isomerization was 50 times and cleavage 76 times as fast as with 3',5'-UpU. Accordingly, isomerization still predominates, being 30 times as fast as cleavage. Interestingly, only migration of the phosphodiester linkage from O3' to O2' was subject to acceleration, not the reverse reaction. Since both isomerization and cleavage underwent acceleration, protonated amino groups evidently enhance formation of the common phosphorane intermediate. The electron density at O3'-linked phosphorus atom is lowered inductively and electrostatically, possibly even by H-bonding. These influences on the O2'-linked phosphorus remain weaker, owing to longer distance. The same influences then reduce more markedly the nucleophilicity of 3'-OH than that of 2'-OH. Altogether, migration form O3' to O2' becomes favored, though the equilibration through pseudorotation still is fast. Cleavage that may take place without pseudorotation, is accelerated even more than isomerization.



Structures 1a/b and 2a/b

Guanylyl-3',3'-(2'-amino-2'-deoxyuridine) (**2a**) is another model compound used for quantification of influences that amino functions may exert on the cleavage of RNA phosphodiester linkages under physiological conditions.[59] The predominant ionic form of **2a** is at pH 5–12 (90 °C) mono-anion that undergoes a pH-independent cleavage to guanosine 2',3'-cyclic monophosphate and 2'-amino-2'-deoxyuridine at pH 6–8. Reaction is 250 times as fast as the corresponding reaction of its 2'-OMe analog **2b**.[59,60] Interestingly, **2a** does not undergo isomerization, in striking contrast to 3',5'-UpU. Evidently, the phosphorane intermediate/transition state is not sufficiently stable to pseudorotate. This is the case when the reaction proceeds through a minor tautomer having the 2'-OH proton shifted to the 2'-amino group, as depicted in Scheme 5. The pK_a values of the 2'-hydroxy and 2'-ammonium groups differ by 7 units, which means that the mole fraction of the mono-anionic zwitterion is only of the order of 10^{-7}. 2'-Oxyanion, however, is a much more powerful nucleophile than 2'-hydroxyl group. As regards attack on a neutral phosphotriester, the difference is around 9 orders of magnitude, as seen from the fact that isomerization uridine 3'-dimethylphosphate turns hydroxide ion catalyzed already at pH 2.[61] The difference may well be smaller as attack on a phosphodiester mono-anion is concerned. In the present case, H-bonding of the 2'-ammonium group to a non-bridging phosphate oxygen, however, reduces the electron density on phosphorus. In addition, protonation of the 2'-amino group inductively improves the leaving group. Attack of 2'-O^- on the H-bonded phosphodiester mono-anion gives a di-anionic phosphorane that evidently is too unstable to pseudorotate.

In conclusion, an amino group when brought close to a scissile phosphodiester linkage may greatly affect the course



Scheme 5. Cleavage of guanylyl-3',3'-(2'-amino-2'-deoxyuridine) (**2a**) via a minor tautomer.

and kinetics of its reactions by electrostatic effects and possibly by participation in proton shuttling.

2.2. Studies with oligoribonucleotides

One may question how well the mechanistic conclusions based on kinetic behaviour of dinucleoside-3',5'-monophosphates are applicable to cleavage of polynucleotides. While this certainly still is an open question, measurements with poly U suggest that the difference between dimeric and polymeric phosphodiesters is not fundamental. The essential features of the pH-rate profiles for cleavage and isomerization of phosphodiester linkages within poly U are rather similar to those of 3',5'-UpU.[32] The pH-independent isomerization and the hydroxide-ion-catalyzed cleavage both are as fast as with 3',5'-UpU. The only significant difference is that the hydronium-ion-catalyzed cleavage is one order of magnitude faster than with 3',5'-UpU. Accurate determination of the rate constant for the pH-independent cleavage is prone to errors, but this reaction also seems to be somewhat faster than with 3',5'-UpU. Anyway, the pH-independent isomerization still predominates with poly U, being 5 times as fast as the cleavage.

However, the kinetic data obtained with poly U do not adequately represent the situation with natural RNA. The hydrolytic stability of RNA phosphodiester bonds strongly depends on the molecular environment.[62] While the influence of base moiety structure on cleavage or isomerization of dinucleoside-3',5'-monophosphates is under physiological conditions rather modest,[31] studies with natural RNA oligomers[63–66] and synthetic oligonucleotides[67–69] have shown that 3',5'-UpA, 3',5'-UpG, 3',5'-CpA and 3',5'-CpG linkages often are exceptionally labile. In other words, a combination of 3'-linked pyrimidine and 5'-purine nucleoside constitutes a favourable cleavage site. Kinetic studies with 2'-*O*-methylated oligonucleotides that contain only one non-methylated nucleoside and, hence, only one scissile phosphodiester bond, however, challenge the simple nearest-neighbour concept as a sufficient description for the influence of base moiety composition on RNA stability.[7] Base composition or base sequence at a distance of several nucleotides from the scissile bond seems to be of equal importance as the identity of the nearest bases. Among dodecamers that all contained a 3',5'-UpA as the cleavage site, the cleavage rate at pH 8.5 (CHES buffer at 35 °C) relative to the cleavage at the same site within a 5'-AUAA-3' tetramer varied from 15-fold retardation to 15-fold acceleration. None of these oligonucleotides showed by MFOLD, a web server for nucleic acid folding and hybridization prediction, or melting experiments any tendency for formation of intra- or inter-chain secondary structures. It is also important to note that this very marked variation in the cleavage rate of the 3',5'-UpA site at pH 8.5 largely disappears on going to pH 12. Another noteworthy observation is that

the base sequence required for maximal cleavage rate depends on the identity of the nearest neighbours, being different with 3',5'-UpA and 3',5'-CA sites.

Molecular dynamics simulations at 2.1 and 3.0 ns scale have helped to rationalize the experimental observations discussed above.[70,71] Stacking of nucleobases that largely determines the overall structure of a single stranded oligonucleotide in aqueous solution,[72] is context-dependent.[73] In other words, stacking tendency of bases at several nucleotides distance from the scissile phosphodiester bond still affects stacking of the bases at the cleavage site. Each individual stacking process within the chain must compromise with the stacking demand of bases further away. Stacking geometries within nucleic acids differ from the structures of isolated stacked dimers.[74] Accordingly, stability of a given phosphodiester bond is sequence-dependent, not only nearest neighbor-dependent. Enhanced stacking increases rigidity around the scissile phosphodiester bond. This, in turn, retards reorientation towards a co-linear orientation of P–O2' and P–O5' bonds that is a prerequisite for PO-bond cleavage. In other words, increased rigidity due to strong stacking in the vicinity of the scissile bond explains why a given dinucleotide fragment is cleaved within an oligonucleotide less readily than its unconstrained small molecule counterpart.

It is more difficult to envisage why the cleavage of some dinucleotides when introduced into an oligomer, experience a rate acceleration. Molecular dynamics simulations of oligonucleotides that contain in place of the scissile phosphodiester bond a phosphorane having 2'-O and 5'-O in apical position offer a tentative explanation.[71] While the stacking interaction between the 3'- and 5'-linked nucleosides is lost on going to the transition state, stacking of the remaining phosphodiester bonds may strengthen. Depending on sequence, this strengthening can even be sufficiently substantial to overcompensate the loss in overall stacking energy caused by unstacking around the scissile bond. In other words, the remnant (3'-linked) and departing (5'-linked) oligomer, become stabilized compared to the initial state. Part of the product stabilization is present already in the transition state, resulting in a rate-acceleration compared to a system, such as poly U, where stacking interactions are negligible.

Consistent with the preceding discussion on the influence of base stacking, the initial state conformation around the scissile phosphodiester bond plays on important role. The closer the initial state mimics the transition state, *i. e.* the better O2', P and O5' fall on the same straight line, the easier is transition from initial to transition state, owing to minimal changes in stacking interactions.[75] Breaker's group has estimated the effect of initial state conformation on the cleavage rate to be at least 100-fold.[76]

Besides base stacking, sugar ring puckering influences on initial state structure. The predominant ring puckering in RNA is C3'-*endo* (N),[77–79] but recent studies with 5'-UU^{mod}A-3' trinucleotides containing a locked N-type 6,3'-methanouridine or S-type 4'-methyluridine as the intervening nucleoside, suggest that the reaction through the S-conformer is considerably faster. In other words, barrier for the conversion of initial sate to an in-line type transition state is lower with the C2'-*endo* (S) conformer than with the predominating C3'-*endo* (N) conformer.[80] Accordingly, nucleotides that for some structural reason are constrained to S conformation may undergo an exceptionally fast phosphodiester bond cleavage. In the absence of any substantial constrain, the N=S isomerization evidently is, according to Curtin-Hammett principle, too fast to affect the cleavage rate.

Besides linear structures, single stranded oligonucleotides are present in hairpin loops and bulges. 2'-*O*-Methylated oligonucleotides containing one non-methylated nucleoside in the loop[81] or bulge[82] behave as their linear counterparts discussed above. In other words, 10-fold accelerations and retardations compared to linear oligo U occur at pH 8.5. In the middle of a pentanucleotide loop or bulge, the cleavage rate is comparable to that of a linear structure. Closer to double helix, the cleavage is somewhat slower. A 2',5'-linkage is cleaved within a hairpin loop as readily as 3',5'-linkages.[81]

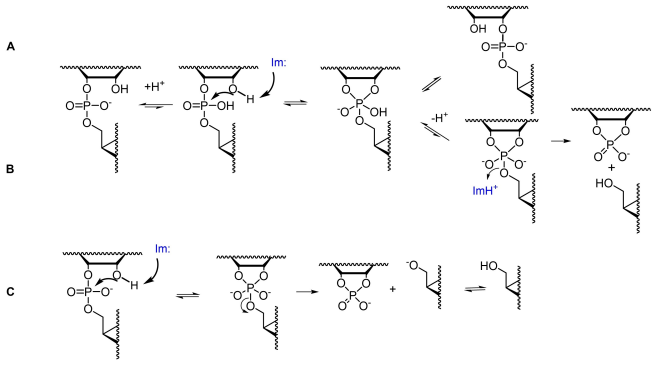
One factor that still may crucially influence on the cleavage rate is H-bond network around the cleavage site that mediates proton transfer from the attacking 2'-OH to the phosphorane intermediate and/or from the phosphorane intermediate to the departing 5'-oxyanion.[67] The rate variation between various phosphodiester bonds depends on pH, being much more prominent in CHES buffer (pH 8.5)[7] than in millimolar concentrations of hydroxide ion.[68] Presumably, proton transfer by solvent water from the attacking 2'-OH to departing O5' plays a more important role at pH 8.5 than under more basic conditions where the reaction via a highly unstable dianionic phosphorane evidently predominates. Unfortunately, almost all experimental data available refer to rather high pH.

3. General acid/base-catalyzed reactions of RNA phosphodiester linkages

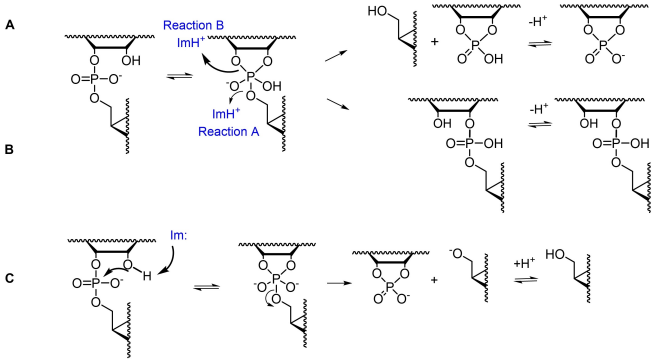
Cleavage of RNA phosphodiester linkages by buffer constituents has been a subject to considerable interest since late 1980s, mainly as a model of RNAse A catalysis where two imidazole residues of histidine play a crucial role. According to the pioneering studies of Breslow, the buffer-catalyzed cleavage proceeds in parallel with isomerization via a common phosphorane intermediate.[33,83] The mechanistic interpretation was that imidazolium ion catalyzes formation of the intermediate by a specific acid/general base mechanism. In other words, the mono-anionic phosphodiester group is protonated in a rapid pre-equilibrium step and imidazole base catalyzes by

proton abstraction the attack of 2'-OH on the neutral phosphodiester linkage (A in Scheme 6). The mono-anionic phosphorane intermediate obtained is sufficiently stable to pseudorotate giving the 2',5'-isomer without additional catalysis. Isomerization, hence, is susceptible to general acid catalysis by imidazolium ion. Cleavage, in turn, takes place by a specific base/general acid pathway, i.e. by pre-equilibrium deprotonation of the mono-anionic phosphorane to di-anionic species and subsequent imidazolium ion catalyzed departure of the 5'-linked nucleoside (B in Scheme 6). The unusual feature of this mechanism is that both formation and breakdown of the phosphorane intermediate are subject to experimentally observable general acid/base catalysis. The mechanism has received criticism,[84–86] to which Breslow has responded by additional measurements,[87] and admitted that although the original mechanistic description is valid, a simple general base catalyzed reaction via a di-anionic phosphorane may occur in parallel (C in Scheme 6).

Consistent with the experimental data of Breslow, Kirby has reported that the cleavage reaction of a pentanucleotide model, 5'-TTUTT-3', is susceptible to catalysis by both



Scheme 6. Breslow's mechanism for the cleavage of RNA phosphodiester linkages by imidazole buffers.



Scheme 7. Kirby's mechanism for the cleavage of RNA phosphodiester linkages by imidazole buffers.

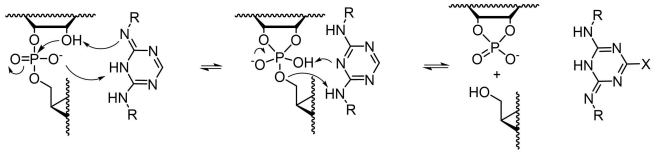
imidazole and imidazolium ion, the contribution of general acid catalysis being more important.[34] Imidazolium ion, in turn, catalyzes the isomerization. The mechanistic interpretation is based on competition of two reaction pathways: (i) a rapid pre-equilibrium formation of a mono-anionic phosphorane, followed by rate-limiting general acid-catalyzed cleavage (A in Scheme 7) and general acid-catalyzed isomerization (B in Scheme 7) and (ii) a general base-catalyzed cleavage via a di-anionic phosphorane intermediate (C in Scheme 7).

A fact that markedly complicates interpretation of the experimental data is that catalysis by imidazole buffers is rather inefficient compared to the buffer-independent reactions. The experimental data typically refers up to molar buffer concentration, which makes complete elimination of salt and co-solute effects challenging. Above all, the contribution of buffer catalysis to the observed rate of isomerization is so small that distinguishing of general catalysis from medium effects is susceptible to misinterpretation. In 0.7 molar buffer solution, the acceleration is only 30–80 %, depending on the buffer ratio.[34] Regardless of which one of the two plausible mechanisms discussed above is a better description for the cleavage of RNA phosphodiester bonds one thing is indisputable. Buffer catalysis markedly influences on the partition of the phosphorane intermediate to the cleavage and isomerization products. With 5'-TTUTT-3', the buffer-independent isomerization is at pH 5.85 (80 °C, $I = 0.5$ M) 27 times as fast as cleavage, whereas at buffer concentration 0.7 M, cleavage in 4 times as fast as isomerization.[34] As discussed in the previous chapter, endocyclic fission of the phosphorane intermediate is a more facile process than the exocyclic cleavage. Imidazolium catalysis predominantly takes place where most needed, i.e. to accelerate exocyclic departure.

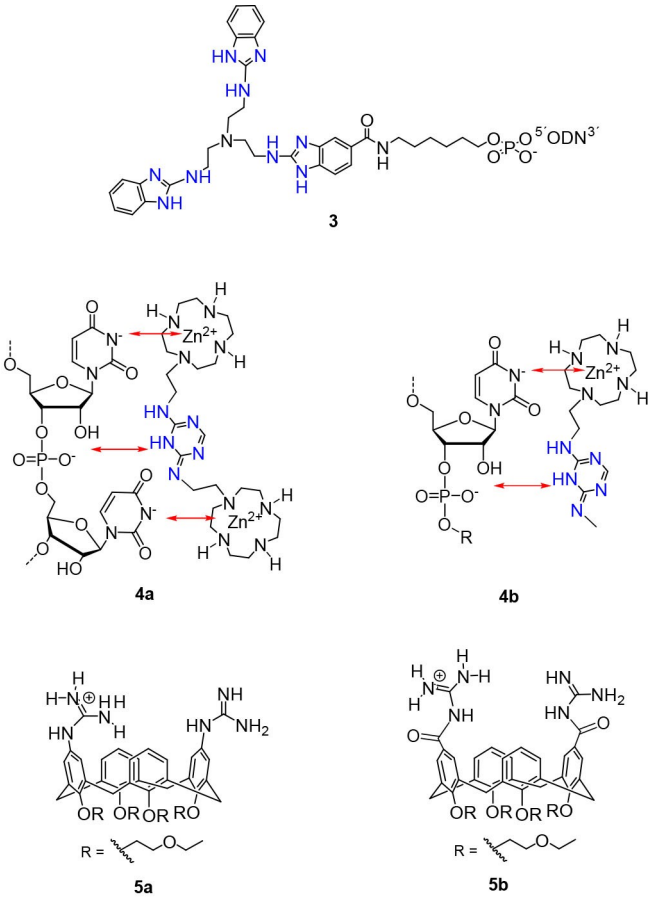
As discussed in chapter 2.1., the un-catalyzed pH-independent cleavage of RNA phosphodiester linkages most likely is a two-step reaction where proton shuttling plays an important role, first from the attacking 2'-OH to the developing phosphorane and then from the phosphorane to the departing 5'-oxygen. Guanidine group has received interest as an agent that could mediate proton shuttling via different tautomeric forms.[88] It is the side-chain functionality of arginine, occurring in the catalytic center of some nucleases[89,90] and as a substructure of catalytically active guanine base in hammerhead[27,91] and hairpin[92] ribozymes.

Guanidine derived compounds shown to cleave phosphodiester linkages typically contain two guanidine groups. The factors that are of importance for efficient proton shuttling, in addition to overall structure of the cleaving agent, include pK_a value close to 7 of the guanidinium units, planar hydrophobic structure and small free energy difference between the tautomeric forms.[93] The pioneering study in the field of nonmetallic cleaving agents of RNA is the finding of Göbel's group[94,95] that tris-guanidines derived from 2-aminobenzimi-

dazole units (**3**) are effective catalysts. The mechanism of cleavage, however, remained unclear. More recent studies with 2,4-diamino-1,3,5-triazine have shed some light on the catalytic action of guanidine. This compound consists of a fusion of two guanidine fragments with one common nitrogen atom that allows proton transfer via tautomeric equilibration. For this purpose, 2,4-diamino-1,3,5-triazine was anchored close to the phosphodiester linkage of 3',5'-UpU by tethering the Zn^{2+} complex of cyclen to its primary amino groups (**4a**).[96] The well-established high affinity binding of the two Zn^{2+} complexes to uracil bases[97,98] that anchors agent **4a** to 3',5'-UpU resulted in 100-fold rate acceleration in the phosphodiester cleavage reaction at pH 6 (90 °C).[96] The rate remained pH independent at pH 6–8, but at higher pH, catalysis by hydroxide ion took over. Isomerization was still observable at pH 6–7, but **4a** did not catalyze the reaction. The 1,3,5-triazine core is neutral at pH > 6 (pK_a = 3.96 at 25 °C), as well as its less basic 6-OMe (pK_a = 3.54) and more basic 6-NHMe substituted (pK_a = 5.28) counterpart.[99] All three cleaving agents behaved similarly. In other words, the catalysis was not susceptible to the basicity of the cleaving agent. According to the authors, proton shuttling via various tautomeric forms of the 1,3,5-triazine unit explains the insensitivity (Scheme 8). Upon formation of the phosphorane intermediate, the 2,4-diaminotriazine serves both as a general base abstracting a proton form 2'-OH and a general acid donating a proton to a non-bridging oxygen ligand of phosphorus. Upon breakdown of the intermediate, 1,3,5-triazine then abstracts a proton from the phosphorane hydroxyl ligand and donates it to the departing 5'-O. In other words, 1,3,5-trazine mediates proton transfer from the attacking 2'-OH to the developing phosphorane intermediate and from the intermediate to the departing nucleoside. Since 1,3,5-triazine core in both steps has a role of both a general base and a general acid, the influence of its overall basicity remains negligible. Cleaving agent **4b** bearing only one anchoring arm also catalyzes the cleavage of UpU, although somewhat less efficiently than **4a**, owing to less efficient complex formation. This one-armed cleaving agent allowed determination of the leaving group effect with uridine 3'-alkylphosphates.[100] The $\beta_{lg} = -0.7$ is comparable to the one, $\beta_{lg} = -0.59$,[39] reported for the pH- and buffer-independent cleavage that was suggested to proceed by proton shuttling.



Scheme 8. Cleavage of RNA phosphodiester linkages by proton shuttling via various tautomeric forms of 2,4-di(alkylamino)-1,3,5-triazines.



Structures 3, 4a/b and 5a/b.

Calix[4]arenes bearing two guanidine groups in 1,3-distal position constitute another set of efficient nonmetallic RNA cleaving agents.[101] In this case, the optimal pH, however, is around 10 in 80 % aq. DMSO, *i.e.* under conditions where the cleavage proceeds by a hydroxide ion catalyzed mechanism in the absence of any cleaving agent. At this pH, one of the guanidine groups is neutral, the other protonated (**5a**). When the guanidine groups were attached to the calix[4]arene scaffold via a carbonyl group (**5b**), the optimal pH dropped to 9.3.[102] A general base/general acid mechanism has been suggested, the neutral guanidine deprotonating the attacking 2'-OH and the protonated one donating the proton to phosphate oxy ligand. In other words, the contribution of general acid catalysis refers to facilitation of the nucleophilic attack on phosphorus, not facilitation of departure of the leaving group by protonation. The group had previously proposed this mechanism for the cleavage of 2-hydropropyl *p*-nitrophenyl phosphate having a very good leaving group.[103] With a nucleosidic leaving group, facilitation of the departure of nucleoside 5'-oxyanion by proton transfer to O5' appears

more attractive. The latter mechanism also explains why **5a** catalyzes the departure of a poor nucleosidic leaving group much more effectively than the departure of *p*-nitrophenoxide ion.

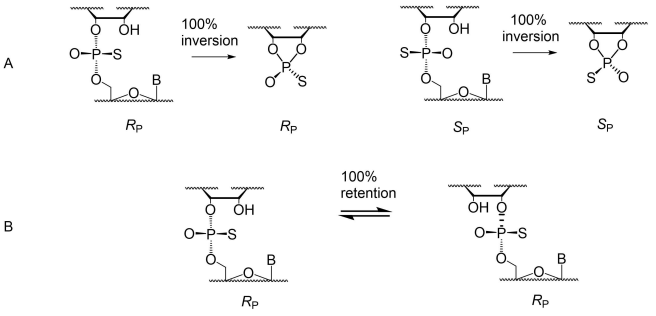
4. Cleavage of phosphorothioate, phosphoramidate and phosphonate analogs of RNA phosphodiester linkages

Replacement of one or two of the phosphoryl oxygen atoms with sulfur, nitrogen or carbon has been of interest, owing to the extensive use of such oligonucleotides in mechanistic enzymology and in development of oligonucleotide based drugs. The discussion below tends to summarize the results of experimental and theoretical studies undertaken to obtain a solid chemical basis for such applications.

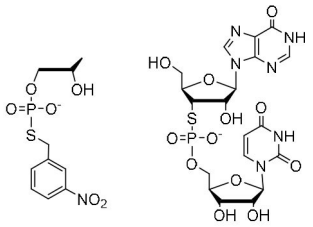
Phosphorothioate and phosphorothiolate diesters. Substitution of a non-bridging oxygen atom of a phosphodiester linkage with sulfur has received interest since the R_P and S_P diastereomeric phosphorothioate linkages serve as stereochemical probes for Mg^{2+} binding in mechanistic studies of ribonucleases and ribozymes. As a hard metal ion, Mg^{2+} binds poorly to sulfur. A rate retardation observed upon replacement of a particular oxygen atom with sulfur, hence, indicates that this oxygen participates in metal ion binding. Replacement of Mg^{2+} with a soft metal ion, such as Mn^{2+}, Zn^{2+} or Cd^{2+}, should then restore the catalytic activity, at least partially. In the absence of metal ions, the stereochemistry of non-bridging thio-substitution is less important. When the reaction proceeds via a di-anionic phosphorane (pH > 9), the thio effect k_O/k_S is 1.3 and 0.8 for the R_P and S_P diastereomer, respectively (k_O and k_S refer to rate constants for phosphodiester and phosphorothioate, respectively).[37] With the dithioate analog, the thio effect is slightly more rate-retarding, $k_O/k_S = 2.8$.[104] The transition state structure seems to resemble that of phosphodiesters, since the leaving group effects are very similar: $\beta_{lg=} -1.23 \pm 0.03$ and -1.24 ± 0.03 for R_P and S_P isomers of uridine 3'-(alkylphosphorothioate)s[105] are within the limits of experimental errors equal to $\beta_{lg=} -1.28 \pm 0.05$ of uridine 3'-alkylphosphates.[38] Because sulfur is a soft easily polarizable element compared to oxygen, one might expect that thio-substitution stabilizes the di-anionic transition state, as also indicated by QM/MM[106] and DFT[107,108] calculations. Owing to lower charge density of sulfur, the transition state, however, is less efficiently solvated, and the two opposite effects nearly counterbalance each other.

When the reactions take place by a pH-independent mechanism via a mono-anionic phosphorane intermediate (pH < 8), the effect of non-bridging thio-substitution is entirely different. The predominant reaction is desulfurization, being responsible for 80% of the conversion of 3',5'-Up(s)U to immediate products, viz. 2',5'- and 3',5'-UpU, 2',5'-Up(s)U, and 2',3'cUMP(s).[37] The cleavage expectedly proceeds by 100% inversion and isomerization by 100% retention of configuration around phosphorus (Scheme 9). According to DFT calculations, the thiophosphorane intermediate is approximately as stable as oxyphosphorane.[107] It evidently is stable enough to allow protolytic equilibration between the non-alkylated oxy and thio ligands, as well as the accompanying pseudorotation that then enables departure of the SH ligand as hydrogensulfide ion. One has to bear in mind that SH^- is a much better leaving group than OH^-; H_2S is 5 orders of magnitude stronger acid than H_2O. As regards the cleavage to 2',3'cUMP(s) and isomerization to 2',5'-Up(s)U, the cleavage is 3–9 times as fast as with 3',5'-UpU and the isomerization rate varies from 13% to 20% of that of 3',5'-UpU.[37,104] The reason for markedly retarded isomerization most likely is facile desulfurization that competes of the thiophosphorane intermediate.

Phosphorothiolate oligonucleotides having either the bridging O3' or O5' replaced with sulfur are useful mechanistic probes of enzyme catalysis. Thionucleoside 5'-sulfide ion is a much better leaving group than nucleoside 5'-oxyanion, accelerating the hydroxide-ion-catalyzed cleavage via dianionic thiophosphorane by a factor of 10^4- 10^5.[109,110] Heavy atom isotope effects $^{18}k_{nuc} = 1.0245 \pm 0.0047$ and $^{34}k_{lg} = 1.0009 \pm 0.0001$ for the cleavage of *O*-(2-hydroxypropyl) *S*-(3-nitrobenzyl) phosphorothiolate (**6**), a simple model of a dinucleoside-3'O,5'S-phosphorothiolate, are consistent with an early transition state where formation of the P–O bond is far advanced without appreciable lengthening of the P–S bond.[111]



Scheme 9. Stereochemistry for the cleavage (A) and isomerization (B) of RNA phosphorothioate linkages.



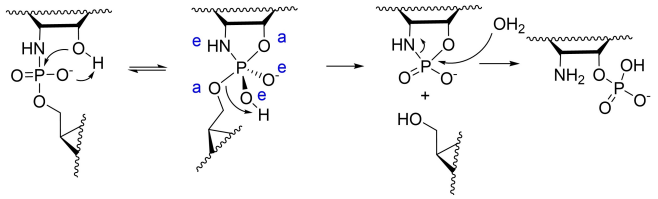
6 7

Structures 6 and 7.

Bridging S3' substitution, in turn, results in a 100-fold acceleration,[112,113] that evidently is of dual origin. Theoretical calculations suggest that the 5-membered ring formed upon the attack of 2'-OH is not as strained as with O3' nucleosides and high polarizability of sulfur additionally stabilizes the transition state.[114] At pH < 7, isomerization starts to compete with the hydroxide-ion-catalyzed cleavage and gradually takes over, being the only reaction detected at pH 4–6.[115] With 3'-deoxy-3'-thioinosinylyl-3',5'-uridine (**7**), this pH-independent isomerization is around 50 times as fast as the pH-independent isomerization of 3',5'-UpU. Importantly, isomerization of **7** is not reversible. In other words, the 3'-mercapto function is unable to attack phosphorus.

Phosphoramidate derived diester analogs. Substitution of the 3'-oxygens in oligonucleotides with nitrogen increases the resistance of both DNA[116,117] and RNA[118] oligomers toward nucleases without losing the efficiency and selectivity of hybridization. Their thiophosphoramidate analogs have received interest in chemotherapy, owing to their ability to inhibit the telomerase enzyme expressed in many cancer cells.[119]

Mechanisms for the cleavage of internucleosidic 3',5'-phosphoramidate linkage have been studied with 3'-amino-3'-deoxyuridyl-3',5'-uridine.[120] The predominant reaction under neutral conditions (pH 6–8 at 90 °C) is pH-independent cleavage of the P–O5' bond giving 3'-amino-3'-deoxyuridine 2'-monophosphate and uridine (Scheme 10). In all likelihood, 2'-OH attacks on mono-anionic phosphoramidate concerted with proton transfer from the attacking oxygen to a non-bridging phosphoramidate oxygen. The departing 5'-O adopts



Scheme 10. pH-independent cleavage of RNA phosphoroamidate linkages.

an apical position upon formation of the pentacoordinated intermediate and undergoes a rate-limiting departure concerted with proton transfer from the hydroxyl ligand of the intermediate to 5'-O. The pseudorotation barrier evidently is too high to allow N3' to take an apical position and, hence, leave resulting in isomerization to a 2',5'-phosphodiester. The O2',N3'-cyclic phosphoramidate obtained then undergoes a kinetically invisible post-transition state hydrolysis to 3'-amino-3'-deoxyuridine 2'-monophosphate. Consistent with the facile cleavage of the P–N bond, 2'-amino-2'-deoxyuridine N2',O3'-cyclic phosphoramidate also reacts exclusively by the P–N bond cleavage at pH 6.[110]

Under more acidic conditions (pH < 6), cleavage of the P–$N^{3'}$ bond gradually becomes favored, giving 3'-amino-3'-deoxyuridine and uridine 5'-phosphate as the final products.[120] Under more alkaline conditions (pH > 8), the hydroxide-ion-catalyzed cleavage to 3'-amino-3'-deoxyuridine N3'-phosphoramidate predominates. In other words, the initially formed O3',N3'-cyclic phosphoramidate is evidently hydrolyzed at high pH via P–O2' fission since O2'-oxyanion is a better leaving group than N3'-amide ion.

Phosphonate analogs of phosphodiesters. Substitution of the 5'-oxygen of with carbon affords phosphonate analogs that inhibit enzymes processing phosphoesters.[121] While 5'-*C* substitution effectively prevents cleavage of phosphodiester bonds under neutral conditions, isomerization of O3',C5' diesters to O2',C5' diesters still takes place. Over a wide pH-range from 5 to 9 (90 °C), pH-independent isomerization actually is the only reaction detected.[122]

5. Transition state models for the action of large ribozymes

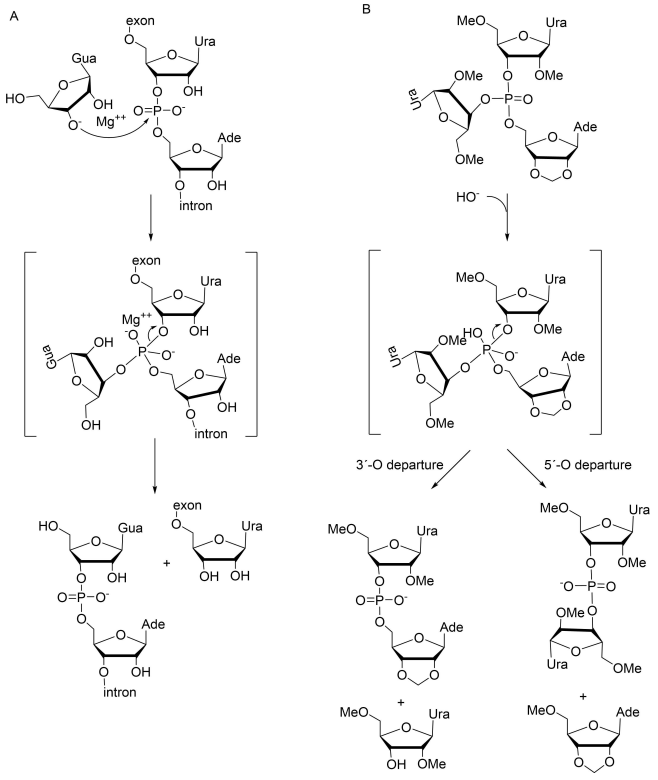
RNA cleavage by the large ribozymes (group I and II introns) differs fundamentally from the reactions catalyzed by ribonucleases and small ribozymes. Instead of the 2'-OH adjacent to the scissile phosphodiester linkage, large ribozymes use as the attacking nucleophile an external nucleoside brought to the reaction center by appropriate folding of the oligonucleotide chain. Another major difference compared to non-enzymatic, nuclease-catalyzed and small ribozyme-catalyzed cleavage is that the leaving oxygen is a 3'-O, not 5'-O,[123–125] and Mg^{2+} facilitates the nucleophilic attack by binding to the non-bridging oxygens of the scissile phosphodiester bond.[126,127]

To clarify the chemical basis for some details of the mechanistically complicated action of large ribozymes, simplified models for the structures obtained by an attack of external nucleoside on a scissile phosphodiester linkage have been prepared and kinetics of their breakdown studied. The underlying idea is that hydrolysis of the phosphotriester obtained by an attack of external alcohol on a phosphodiester is the reverse

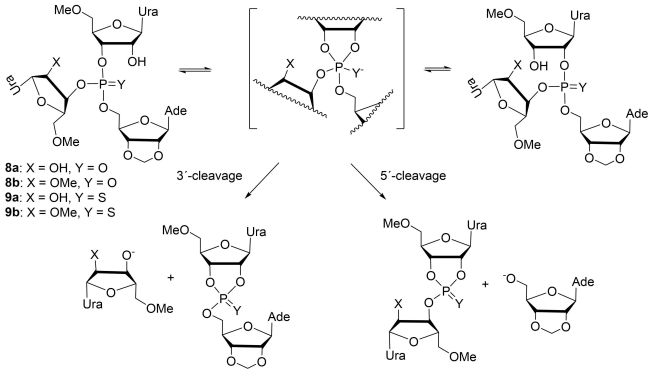
reaction for formation of the same triester. Accordingly, these two reactions proceed through a similar pentacoordinated intermediate/transition state. Scheme 11A shows the reaction catalyzed by Group I introns.[128] The 3'-oxyanion (Mg^{2+} salt) of an external guanosine monomer attacks the scissile 3',5'-phosphodiester linkage giving a Mg^{2+} stabilized di-anionic pentacoordinated intermediate or transition state that breaks down by departure of the 3'-linked nucleoside. For comparison, the mono-anionic intermediate for hydroxide-ion-catalyzed hydrolysis an appropriately protected trinucleoside-3',3',5'-monophosphate is depicted in Scheme 11B. As mentioned above, the reaction of Group I introns proceeds entirely by cleavage of the P–O3' bond. The same reaction also predominates with the trinucleoside-3',3',5'-monophosphate model, but P–O5' fission still competes.[129] 15% of the cleavage takes place by P–O5' fission over the entire temperature range from 3 °C up to 90 °C. When the same trinucleoside core structure was inserted into an oligonucleotide chain, the cleavage rate dropped to one sixth of that of the monomeric triester, and the proportion of P–O5' cleavage turned temperature dependent, being only 3% at low temperature (3 °C) and 20% at high temperature (90 °C).[130] Evidently, base-stacking around the cleavage site retards both P–O3' and P–O5' fissions, but the P–O5' more markedly. In Group I introns, the cleavage site lies within a double helix,[131] where the stacking undoubtedly is stronger than with single stranded oligonucleotide models. Even with a simple trinucleoside-3',3',5'-monophosphate model, elongation of all three arms with short oligonucleotides is sufficient to entirely suppress the P–O5' cleavage.[132]

Another structural feature considered to favor the P–O3' cleavage is stabilization of the departing 3'-oxyanion by H-bonding with the vicinal 2'-OH group.[133–135] Studies with trinucleoside-3',3',5'- monophosphates (Scheme 12) also verify the rate-accelerating effect of the vicinal 2'-OH, but suggest that the influence is based on stabilization of the phosphorene intermediate rather than the departing group stabilization by H-bonding.[136] The hydroxide-ion-catalyzed cleavage of **8a** containing an unsubstituted vicinal 2'-OH was 27 times as fast as that of its 2'-OMe analog **8b**. However, the departure of both the 3'- and 5'-oxyanions was accelerated equally. Accordingly, the acceleration most likely results from stabilization of the common phosphorane intermediate by H-bonding with the neighboring 2'-OH. If this is the case, interconversion of 2'- and 3'-isomers should experience also a similar acceleration as the cleavage reaction. With **8a** and **8b**, the isomerization was too rapid to be studied, but with their phosphoromonothioate analog **9a**, isomerization was an order of magnitude faster than with its 2'-OMe analog **9b**.[137]

Simple trinucleoside monophosphate models self-evidently give an oversimplified picture about the course of large ribozyme catalysis. With ribozymes, the intrinsic H-bonding network at the catalytic core and steric constraints may largely dictate the preferred mechanism. Within an H-bonded network, the 2'-OH at the cleavage site may serve simultaneously as an H-bond donor and acceptor, which makes it a much better H-bond donor. Reactions of guanylyl-(3',3')-(2'-amino-2'-deoxyuridine) under conditions where the amino group is fully protonated could shed some light to the influence of a

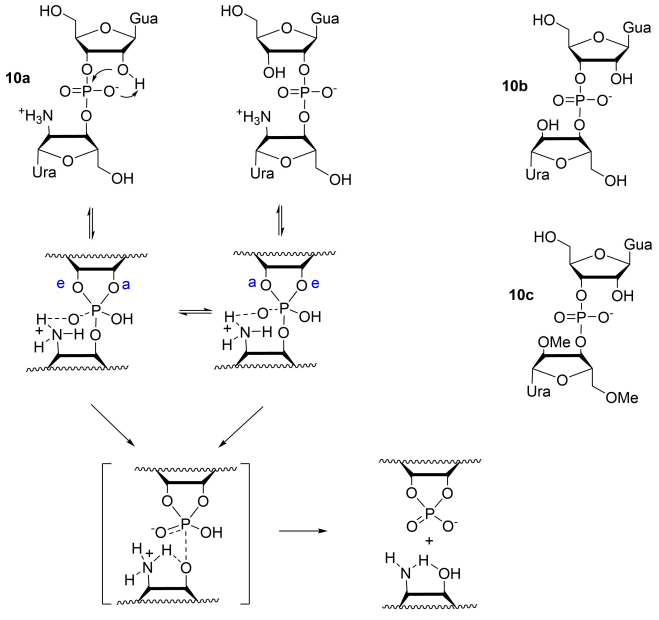


Scheme 11. Comparison of intermediates/transition states for Group I intron self-cleavage (A) and a hydroxide-ion-catalyzed cleavage of a trinucleoside 3',3',5'-monophosphate (B).



Scheme 12. Competition between 3'- and 5'-cleavage in a hydroxide-ion-catalyzed cleavage of trinucleoside 3',3',5'-monophosphates (B).

hydroxyl function exhibiting enhanced acidity. For this purpose, the cleavage of guanylyl-(3',3')-(2'-ammonium-2'-deoxyuridine) (**10a**)[59] was compared to cleavage of guanylyl-(3',3')-uridine (**10b**) and guanylyl-(3',3')-(2',3'-di-O-methyluridine) (**10c**).[60] Spontaneous cleavage of the neutral zwitterionic form of **10a** predominates over a narrow range at pH 3–4 (90 °C).[59] Attack of 2'-OH concerted with proton transfer to a non-bridging phosphoryl oxygen gives a mono-anionic phosphorane intermediate that then breaks down by departure of uridine 3'-oxyanion, most likely concerted with proton transfer from the hydroxyl ligand of the phosphorane intermediate to the leaving oxygen (Scheme 13). The reaction is 16 and 42 times as fast as that of **10b** and **10c**, respectively. In other words, the 2'-ammonium group is clearly more rate accelerating than the 2'-hydroxy function. However, not only cleavage but also isomerization is accelerated, although somewhat less efficiently than cleavage. Compared to isomerization of **10c**, the acceleration is 6-fold. A vicinal ammonium function, hence, seems to stabilize both the phosphorane intermediate and the departing 3'-oxyanion. The 2'-ammonium group evidently stabilizes the phosphorane intermediate by H-bonding and possibly by an electrostatic field effect. As regards the leaving group, inductive electron withdrawal by the neighboring ammonium group additionaly stabilizes the departing oxyanion, or the ammonium group may even serve as an intramolecular general acid.



Scheme 13. Cleavage of neutral zwitterionic guanylyl-3',3'-(2'-ammonium-2'-deoxyuridine).

6. Concluding remark

The cleavage of RNA phosphodiester bonds by RNase A and hammerhead ribozyme at neutral pH fundamentally differs from the spontaneous reactions under the same conditions. While the predominant spontaneous reaction is isomerization of the 3',5'-phosphodiester linkages to their 2',5'-counterparts, this reaction has never been reported to compete with the enzymatic cleavage reaction, not even as a minor side reaction. Most obvious explanation seems to be that the enzymatic reactions proceed via a di-anionic pentaoxyphosphorane intermediate/transition state that is too unstable to pseudorotate, preventing isomerization. The spontaneous process, in turn, takes place via a mono-anionic phosphorane that is able to pseudorotate and, hence, enables isomerization that inherently is more facile than cleavage by the exocyclic PO-bond. Accordingly, the key feature is the efficiency of deprotonation of the attacking 2'-OH. In the absence of an external proton acceptor, a water mediated proton transfer from the 2'-OH to a non-bridging phosphoryl oxygen increases the nucleophilicity of the 2'-OH and electrophilicity of the phosphorus atom, leading to formation of a mono-anionic phosphorane. In case of a biocatalyst, efficient deprotonation of the 2'-OH, in turn, enables attack on the mono-anionic phosphodiester, giving an unstable di-anionic phosphorane intermediate. More or less concerted protonation of the departing 5'-oxygen may still increase the efficiency of catalysis. In case the deprotonation of 2'-OH is less efficient, stabilization of the developing phosphorane by concerted protonation may still enable efficient catalysis but leads to isomerization unless an efficient and highly selective protonation of the 5'-oxygen does prevent it.

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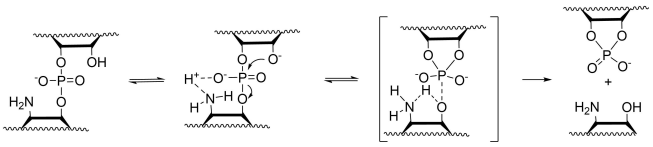

Mechanisms of chemical cleavage and isomerization of RNA phosphodiester bonds discussed as a contribution to conception of a plausible unified picture of bio-catalytic RNA cleavage

*Prof. Dr. H. Lönnberg**