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RNA bulges as targets for selective cleavage by metal ions and organic compounds

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Abstract. The results of studies of RNA bulge structures are reviewed. Cleavage of the phosphodiester bonds within RNA bulges is considered. Attention is focused on the factors influencing the cleavage efficiency. The state-of-the-art of the problem of targeted RNA cleavage induced by various reagents is discussed. The bibliography includes 101 references.

I. Introduction

Most RNA molecules are single-stranded polynucleotide chains with a complex three-dimensional structure (tertiary structure) stabilised through hydrogen bonds between complementary or partially complementary sequences (Watson – Crick complementary base pairing), stacking interactions between loops and binding of loops to metal ions.[1–3] A combination of various interactions gives rise to a unique RNA packaging, which can be compared with a complex protein packaging.

Short double-stranded regions (or stems) and four types of single-stranded regions, *viz*., single-stranded fragments, hairpin loops, internal loops (symmetric or asymmetric) and junctions, are typical elements of the RNA secondary structure.[1]

Bulges are a special case of asymmetric internal loops, *i.e*., RNA regions where the adjacent stems are stacked. Bulges are frequently occurring secondary structure elements. They provide flexibility for the ribose phosphate backbone necessary for RNA folding. RNA bulge loops containing nucleotides that lack complementary bases in the opposite RNA strand play an important biological role. They determine the RNA tertiary structure,[4] represent specific RNA – protein binding sites[5,6] and form active sites of ribozymes.[7,8]

Single-base bulges were studied and characterised in most detail. Single-adenosine bulges most frequently occur in natural RNAs. Bulged adenosine residues (bulge A) serve as specific protein-binding sites, for example, in *E. coli* ribosomal RNAs (5 S rRNA – protein L18 and 16 S rRNA – protein S8)[9,10] and in the operator region of mRNAs encoding the capsid proteins of bacteriophage MS2.[5] Single-uridine bulges were found in many RNAs. For example, this bulge is present in the catalytic site of RNase P,[11,12] in the high-affinity binding sites for the Rev protein to HIV-1 RNA[13,14] and for the Tar protein to HIV-2 RNA.[15] Single-cytosine or -guanine bulges are much more rare; however, such bulges play an important role in organisation of the tertiary structure of natural RNAs.[16,17]

Numerous low-molecular-weight compounds capable of RNA cleavage under physiological conditions (pH 7.0, 37 °C) have been synthesised in recent years.[18–25] RNA cleavage induced by these compounds occurs predominantly at regions that are not involved in secondary structure formation. RNA cleavage within apical hairpin loops has been most extensively studied. RNA cleavage at linkages in bulge loops has been studied to a lesser degree, although bulges have advantages when used as targets for selective RNA cleavage. Both bulges in natural RNAs and tailor-made bulges in RNAs by hybridisation with partially complementary DNA or RNA oligonucleotides can serve as such targets.

II. Structure of RNA bulge loops

1. Methods of investigation of the bulge structure in DNA and RNA duplexes

X-Ray diffraction analysis is most commonly used in studies of DNA and RNA duplex structures.[26–28] In spite of high accuracy of this method, X-ray crystallography of nucleic acids offers only an approximate estimate of general structural parameters of duplexes. It is impossible to construct a satisfactory structural model based on these parameters without invoking data from other methods. Potential energy calculations provide a general insight into internal mobility of RNA molecules. Among spectroscopic methods, NMR is used most often.[29–31] Analysis of nucleic acid structures by NMR spectroscopy provides insight into the molecular dynamic structure in solution and complements X-ray diffraction data, which are somewhat limited due to the static state of the subjects under study. The influence of bulges on the RNA tertiary structure is analysed by gel retardation assay,[32] fluorescence resonance energy transfer (FRET)[33] and transient electric birefringence (TEB).[34] The classification of structural elements of RNAs and their abundance in natural and synthetic RNAs studied by X-ray diffraction and NMR spectroscopy is available through

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the Internet (SCOR: Structural Classification of RNA, http://scor.lbl.gov/index.html).

2. Structures of single-nucleotide bulges

Single-nucleotide bulges appear in RNAs if one nucleotide in the polynucleotide chain lacks a complementary base and is located between two classical Watson – Crick or non-canonical base pairs.[35] This unpaired nucleotide can adopt two main conformations, *e.g.*, can be either looped out of the helix (Fig. 1 *b*) or be stacked into the helix with adjacent bases (Fig. 1 *a*).[36] The conformation of the bulged nucleotide depends on both the nature of the nitrogen base and the adjacent bases. There are examples when the same bulge adopts different conformations, which change depending on intermolecular interactions.

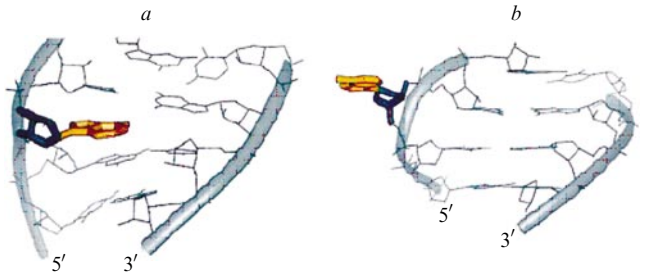


Figure 1. Three-dimensional structure of the RNA double strand containing a single-nucleotide bulge, which is stacked in the helix (*a*) or is looped out from the helix (*b*).[36]

About 30 single-adenine bulges that are present in RNA structures studied by NMR spectroscopy and X-ray diffraction were derived from the SCOR database. In 11 structures, adenosine is located within the helix; in the other structures it is looped out. Generally, the X-ray diffraction method [13, 28, 37–41] shows that in crystals, a bulged adenosine residue is looped out from the RNA helix (Fig. 2), whereas the NMR method [42–45] indicates that this adenosine is located within the helix and is involved in stacking interactions with adjacent bases. Apparently, a bulged adenosine residue is highly mobile, and its position at each instant of time is determined by intra- and intermolecular interactions. Therefore, the discrepancy between the data can be attributed to different conditions in which RNAs exist during experiments: in solution (NMR experiments) or in the solid phase (X-ray diffraction analysis).

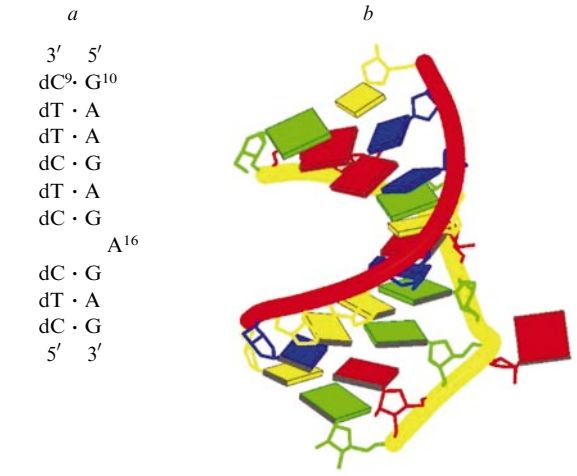


Figure 2. Sequence (*a*) and the three-dimensional structure (*b*) of the RNA · DNA duplex containing a single-nucleotide A bulge.[28]
Hereinafter, G bases are green, A bases, red; C bases, yellow; U bases, dark blue (or pale blue).
The PDB code for the bulge A structure is 1EFO (http://www.rcsb.org/pdb/explore.do?structureId = 1EFO).

In crystals, the duplex structure is stabilised by intermolecular bonds. In addition, intermolecular stacking interactions between flipped-out nucleoside bases located in different duplexes can exist. The looped-out conformation of a bulged adenosine residue in crystals can also be due to the fact that crystallisation is performed in the presence of divalent metal cations, for example, of Mg^{2+}, which can stabilise this structure.[38] NMR spectroscopic studies of duplex structures are performed in solution, where RNA molecules adopt the energetically most favourable conformation. In this conformation, a duplex contains a bulged adenosine oriented inside the helix. As a result of high mobility of a bulged adenosine, different conformations of a bulged adenosine were established in the same RNA duplexes.[27, 46, 47]

To understand which conformation is adopted by a bulged adenosine depending on its environment, an attempt was made to determine the energetically most favourable conformation by computer simulation methods.[48, 49] An hierarchical approach was used [49] to investigate various sterically possible low-energy conformations of single-base bulge loops. The method is based on the assumption that a bulge and adjacent base pairs are responsible for the conformational deformation of the double helix. Computer simulations and calculations of all possible conformations were performed for 6-mer RNA duplexes containing one bulged adenosine. The duplex in which the bulged adenosine lies inside the helix and is involved in stacking interactions with adjacent nucleotides has the lowest energy. Higher Gibbs energies were obtained for RNA duplexes in which the bulged adenosine is located inside the helix and forms a triplex with a base pair at the 5′ end (in the minor groove). According to the calculations, duplexes containing a bulged-out adenosine are characterised by the highest energies. It should be noted that a somewhat different situation was observed for analogous DNA duplexes containing a bulged adenosine. The stacked conformation of a bulged adenosine was also energetically most favourable. However, next in the Gibbs energy was the conformer with a bulged-out adenosine, whereas the conformer in which the bulged adenosine forms a triplex was energetically least favourable.

Single-guanosine bulges (bulge G) have been much less studied. In the structures of all known duplexes containing a bulged guanosine, this nucleoside is looped out from the helix.[16, 50, 51] In natural RNAs, a bulged guanosine was found in the α-sarcin loop region 23 – 28 S of rRNA, where it forms the so-called G-bulged motif.[16] This motif is located in an imperfect stem of a 29-mer hairpin in the $^{5'}$GGAACC· $^{3'}$CAUGACU sequence, where the bulged guanosine (printed in boldface type) is flanked by non-canonical A- -A, C- -C and U- -C base pairs (Fig. 3).

G-Bulged motifs have an important biological function (interactions with α-sarcin and elongation factor G). These motifs were found in the rRNA large subunits of rat and *E. coli* ribosomal RNAs and in the E loop of 5 S rRNA from the frog *Xenopus laevis*.[9, 16, 52–54] The specific structural feature of the G-bulged motif is that the bulged guanosine is flanked by the non-canonical purine-purine base pair A- -A on the 5′ side and forms the G · U · A triplex with the adjacent base pair on the 3′ side (see Fig. 3). This leads to a widening of the major groove of RNA by 5 Å, the groove depth remaining typical of the A-form RNA structure.[16] An analysis of mutant 28 S rRNAs demonstrated that a conserved bulged guanosine, which allows RNAs to fold into a unique tertiary structure, is absolutely

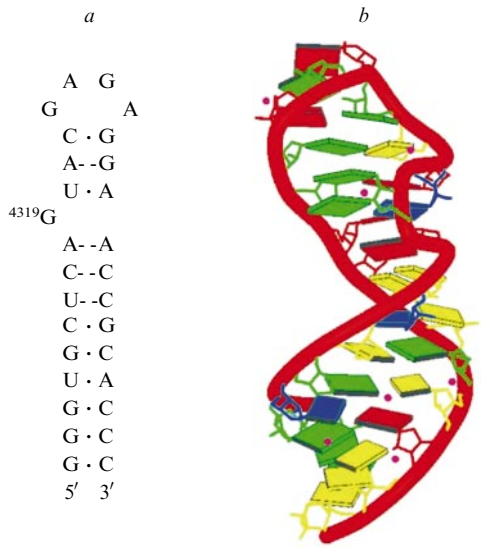


Figure 3. Sequence (*a*) and the three-dimensional structure (*b*) of the α-sarcin loop of the rat 28 S rRNA containing a single-nucleotide G bulge.[16]
The PDB code for the bulge G structure is 430D (http://www.rcsb.org/pdb/explore.do?structureId = 430D).

necessary in the α-sarcin hairpin for rRNAs to exhibit biological activity (binding with the elongation factor).[16]

According to X-ray diffraction data, an uridine residue in single-nucleotide bulges (bulge U) is generally looped out, and it is not only looped out from the helix, but also forms a complex intramolecular structure (for example, it can be directed inward the minor groove).[13, 55] The main distinguishing feature of this conformation of the bulged uridine is that it is stabilised by intramolecular (rather than intermolecular) interactions, to be more precise, by hydrogen bonds between adjacent nucleotides either directly or through aqua bridges. The bulged uridine directed inward the minor groove changes the conformations of adjacent nucleotides. The twist angle of the helix near this bulge increases to 50° (over-twist).[55] The only bulged uridine adopting a stacked conformation inside the helix was found in a ribozyme (Fig. 4).[56] Interestingly, the

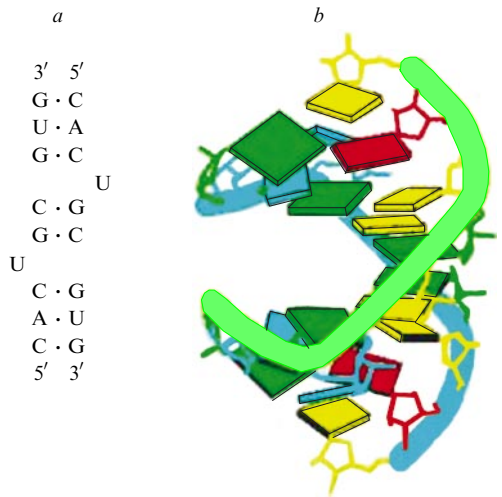


Figure 4. Sequence (*a*) and the three-dimensional structure (*b*) of the duplex containing a bulged uridine.[56]
The PDB code for the bulge U structure is 1J9H (http://www.rcsb.org/pdb/explore/images.do?structureId = 1J9H)

replacement of this uridine by any other nucleoside does not change the activity of the ribozyme, which is not typical of single-stranded regions of catalytic RNAs.[53]

A conserved single-cytidine bulge (bulge C) was found in 5 S rRNA.[17, 57] The structure of this bulge was studied in detail in an 8-mer RNA duplex (Fig. 5) corresponding to the helix II region of 5 S rRNA from the frog *Xenopus laevis*.[51] Crystals of the duplex were studied at 2.2 and 1.7 Å resolution. In both crystal structures, the bulged cytidine is looped out, and the helix axis deviates from the straight line by an angle smaller than 10°. In the crystals studied at 2.2 Å resolution, the bulged base is directed toward the 3′ end of the duplex; in the crystals studied at 1.7 Å resolution, to the 5′ end. The existence of two conformations of the bulged cytidine was attributed to its mobility. In crystal, the bulged cytidine, like the bulged uridine, is involved in several intermolecular hydrogen bonds with nucleotides of the adjacent duplexes as well as in stacking interactions with purine bases.

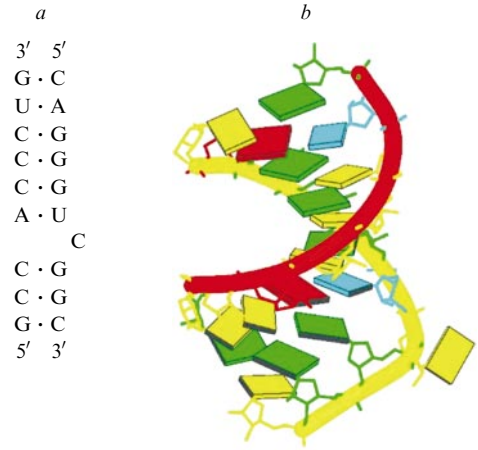


Figure 5. Sequence (*a*) and the three-dimensional structure (*b*) of the duplex containing a bulged cytosine.[17]
The PDB code for the bulge C structure is DQH1 (http://www.rcsb.org/pdb/explore.do? structureId = 1DQH).

The structure of single-nucleotide bulges in RNA · DNA duplexes was established by X-ray diffraction study of the homopyrimidine · homopurine heteroduplex d(C^1TCCTC. .TTC^9) · r(G^{10}AAGAGA^{16}GAG^{19}) containing a bulged adenosine (printed in boldface type) (see Fig. 2).[28] It was demonstrated that the bulged adenosine adopts a looped-out conformation, the ribose ring of the adenosine being almost perpendicular to the helix axis. A comparison of the structure of this duplex with the structure of the RNA · DNA duplex with the same sequence but lacking a bulged adenosine demonstrated that the presence of a bulge has only a slight effect on the bend angle of the helix axis of the RNA · DNA duplex (6°). This behaviour of a bulged adenosine in the RNA · DNA duplex substantially differs from that in RNA · RNA and DNA · DNA duplexes. In crystals, the bulged adenosine (A^{16}) forms the ($C^9 \cdot G^{10}$) · A^{16} triplex with the terminal $C^9 \cdot G^{10}$ base pair of the symmetrically arranged molecule of the heteroduplex through intermolecular hydrogen bonds.

3. Structures of bulges containing two or more nucleotides

As mentioned above, one of the functions of bulges in nature is to organise the RNA tertiary structure, to be more precise, to provide orientation of two adjacent double-stranded RNA regions relative to one another.[1, 58] The influence of the length and the nucleotide composition of a bulge on the RNA

structure was characterised in the studies [32–34, 59] dealing with electrophoretic mobility of bulge-containing RNA·RNA duplexes. An analysis of homo-A and homo-U bulges containing from 1 to 7 nucleotides demonstrated that an increase in the length of the bulge leads to a decrease in electrophoretic mobilities of duplexes,[32] duplexes with U_n bulges always having a higher electrophoretic mobility than duplexes with A_n bulges.[59]

The X-ray data on changes in the bend angle of the axis of a bulge-containing helix were confirmed by the data obtained by the FRET [33] and TEB [34] methods. It appeared that the bend angle depends on two parameters, *viz*., the length of the bulge (an increase in the number of nucleotides in the bulge leads to an increase in the bend angle) and its nucleotide composition (bulges containing primarily purine bases bend the helix axis to a lesser extent than bulges containing pyrimidine bases).[34]

X-Ray diffraction and NMR spectroscopic data demonstrated that bulges consisting of several nucleotides are generally stabilised by a complex system of intermolecular hydrogen bonds, intra- and intermolecular stacking interactions and interactions with metal ions.[38, 51, 60–62] The diversity of nucleotide sequences and the environments of bulges consisting of several nucleotides is responsible for the infinite diversity of their structural forms. The structures of two motifs with particular sequences and structures are known: the above-described G bulges in the α-sarcin hairpin of rRNA and the 'kink-turn' or 'K-turn' structure. The K-turn structure was found for a three-base bulge flanked by two G·C base pairs at the 5′ end and by two or three non-canonical G- -A base pairs at the 3′ end.[51] As a result, the helix axis is bent by 120° at the bulge (Fig. 6). The K-turn motifs play an important biological role. For example, five K-turn motifs were found in 23 S rRNA, and all these motifs serve as binding sites for ribosomal proteins.

Therefore, an analysis of X-ray diffraction and NMR spectroscopic data shows that the looped-out conformation rather than stacked into a helix is more typical of single-nucleotide uridine, guanosine and cytidine bulges. Single-adenosine bulges have higher mobility and can adopt different conformations. Double-stranded RNA regions containing bulges are highly mobile. The major groove of the A helix in the bulge region is widened (predominantly, on the 5′ end of the bulge) due to which it is accessible for interactions with other molecules. In addition, the helix axis is bent at bulges. Numerous multi-membered bulges are known; however, except for the K-turn motif, there are no clear criteria for predicting the three-dimensional structure of bulges with an arbitrary nucleotide sequence.

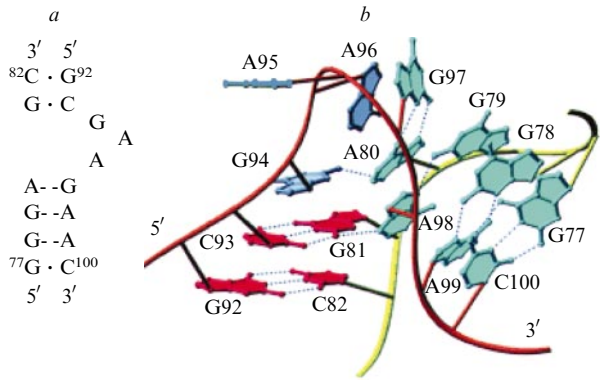


Figure 6. Sequence (*a*) and the three-dimensional structure (*b*) of the K-turn motif of the protein-binding site of 23 S rRNA *Haloarcula marismortui*.[51]
The G·C base pairs at the 5′ end are red, the G·A base pairs at the 3′ end are green and nucleotides in the bulge are blue.

III. Phosphodiester bond cleavage in RNA bulges

1. Geometric parameters of internucleotide bonds and RNA stability

RNA cleavage occurs predominantly in single-stranded regions (hairpin loops, mismatches, internal loops, bulges, junctions and single-stranded fragments). However, it is difficult to determine which bonds and in which sequences are cleaved more rapidly because of complexity of the secondary and tertiary structure of natural RNAs. Many ribozymes and natural and artificial ribonucleases cleave RNA by phosphodiester bond transesterification.[63] It is hypothesised that RNA transesterification can occur only when the attacking oxygen atom of the 2′-hydroxy group of the nucleophile is in-line with the 5′-oxy anion of the leaving group, *i.e*., the arrangement of the leaving group (the oxygen atom of the 5′-hydroxy group), the phosphorus atom and the nucleophile (the oxygen atom of the 2′-hydroxy group) is strictly linear.[64]

Double-stranded RNAs and RNAs involved in RNA·DNA duplexes generally assume an A-form helix, in which the 2′-oxy anion and the 5′-oxy anion of the leaving group are arranged so that the formation of a linear conformation is hindered, resulting in higher stability of double-stranded regions of RNAs and RNA·DNA duplexes compared to single-stranded RNA regions, in which the phosphodiester bonds are labile and can spontaneously adopt an in-line configuration. Therefore, the rate of phosphodiester bond cleavage in RNA bulges would be determined by their ability to adopt an in-line configuration. A phosphodiester bond can spontaneously assume an in-line configuration in the case of sufficient mobility of the corresponding region of the RNA structure. The mobility of a bulge is determined by its size, the nucleotide sequence and the efficiency of intra- and intermolecular stacking interactions between bases.[64]

Nowadays, it is impossible to predict theoretically the sensitivity of the bonds in each particular RNA bulge to cleavage and, much less, to suggest the geometric parameters for the design of a catalytic group capable of performing selective cleavage. In spite of the fact that the specificity toward the RNA structure is always determined for all new compounds exhibiting ribonuclease activity, the dependence of the site and efficiency of cleavage on the RNA primary and secondary structure was studied systematically mainly for divalent metal ions.[65–70]

2. RNA cleavage at bulge sites by metal ions

RNA cleavage by metal ions or metal ion complexes can occur either by the transesterification mechanism [71, 72] or by metal-dependent redox reactions.[73, 74] Many metals catalyse cleavage of internucleotide phosphodiester bonds in RNAs.[75, 76] Initially, oligomers containing a 2′,3′-cyclophosphate at the 3′ end are formed and these oligomers are then hydrolysed to give a mixture of 2′- and 3′-phosphates (Zn^{2+} ions) [77] or only 3′-phosphate (for example, in the presence of Pb^{2+} ions).[78]

Among divalent metal ions, lead ions most efficiently cleave RNA under physiological conditions.[79] Cleavage of various oligoribonucleotides containing bulges of different length by lead ions (Fig. 7 *a*) was analysed.[67] The oligoribonucleotides differed not only in the length, nature and nucleotide sequence of bulges, but also in flanking bases. Adenine bulges flanked by G·C base pairs on both sides are not cleaved by Pb^{2+} ions. The replacement of the G·C base pair on the 5′ side by the U·A or U·G base pair facilitates phosphodiester bond cleavage in bulges. This fact was attributed to the difference in staking interactions between a bulged base and flanking bases. The efficiency of stacking interactions decreases in the series

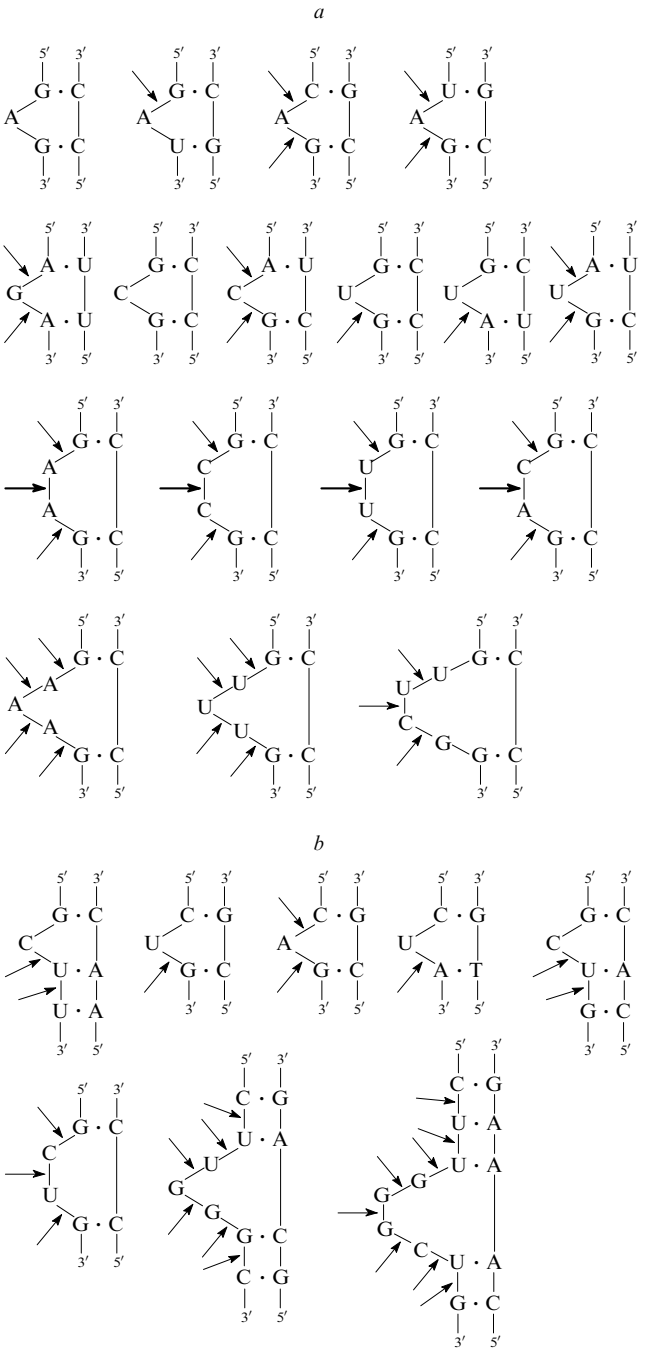


Figure 7. Sensitivity of the phosphodiester bonds in RNA bulges to cleavage by lead ions (*a*)[67] and magnesium ions (*b*).[68]
The arrow thickness corresponds to the cleavage efficiency of a particular phosphodiester bond.

Pu·Pu > Py·Pu > Py·Py,[80] the base on the 5′ side of the bulge making the largest contribution. If a base in a bulge takes part in stacking interactions with adjacent bases, the phosphodiester bonds of the nucleotide in the bulge adopt a configuration similar to that in double-stranded RNA and become slightly sensitive to cleavage.

All known oligoribonucleotides with two-membered bulges (AA, CC, UU and CA) located between two G·C base pairs were cleaved by Pb^{2+} ions, all three phosphodiester bonds in the bulge being cleaved. The bond in the middle of the bulge was cleaved most efficiently. The same situation was observed for three-membered bulges. Phosphodiester bonds in bulges and hairpin loops containing the same nucleotide sequences substantially differ in sensitivity to cleavage by metal ions. For example, the UUCG sequence in an apical hairpin loop is stable and does not undergo cleavage, whereas this sequence in a bulge is readily cleaved by lead ions.[67]

The sensitivity of the phosphodiester bonds in RNA bulges to cleavage by magnesium ions was studied.[68] The formation of bulges composed of 1–4 nucleotides in the 5′-end region of a 132-mer RNA was induced by hybridisation with oligodeoxyribonucleotides. Magnesium ions at a concentration of 2.5 mmol litre^{-1} in weak alkaline solutions (pH 8.0) at 60 °C can cleave RNAs at single-stranded regions,[68] all bulges in RNA·oligonucleotide complexes being cleaved (Fig. 7 *b*).

In single-nucleotide bulges, cleavage occurs predominantly at the phosphodiester bonds on the 3′ side for bulged nucleotides, whereas cleavage of these bonds on the 5′ side for bulged nucleotides is less efficient. The appearance of secondary cleavage sites on the 5′ side for bulges was attributed to the increased flexibility of the bulged nucleotide after cleavage of the most sensitive bond (primary cleavage). When a bulge is flanked on the 3′ side by A·U(T) base pairs, secondary cleavage sites are observed also at sites involved in binding of oligodeoxyribonucleotides, which is attributed to stability of heteroduplexes with a single cleavage site.

Unlike lead ions, magnesium ions cleave all single-nucleotide bulges with the same efficiency independently of the type of the bulged base and the flanking base pairs. When the length of an artificial bulge is increased up to 2–4 nucleotides, primary RNA cleavage at the phosphodiester bond on the 3′ side of the bulge occurs simultaneously with extensive cleavage of the analogous bond in the double-stranded region adjacent to the bulge on the 5′ side. It should be noted that phosphodiester bond cleavage in artificial bulges is accompanied by cleavage of these bonds in all other single-stranded regions in a 132-mer RNA. A comparison of phosphodiester bond cleavage in RNA bulges with cleavage in artificial mismatches demonstrated that, as opposed to bulges, cleavage of sequences flanking mismatches is induced.[68]

The study[81] dealt with phosphodiester bond cleavage by zinc ions in 1–5-nucleotide bulges of a 2′-*O*-methyl oligoribonucleotide, in which only one nucleotide was not modified at 2′ position. Since cleavage of 2′-*O*-methyl oligoribonucleotides occurs not by the transesterification mechanism, the use of such model RNAs allowed the authors to analyse the sensitivity of each particular phosphodiester bond in bulges. The most efficient cleavage was observed for the phosphodiester bond formed by an unmodified uridine residue (rU), which was located in the middle of a five-base bulge. This bond is cleaved two times more rapidly than the analogous bond in a single-stranded oligoribonucleotide. Four- and three-nucleotide bulges containing riboadenosine or ribouridine in the apical position are cleaved by zinc ions at approximately the same rate. In the case of single- and two-nucleotide bulges, the cleavage rate sharply decreases.

The computer analysis[81] of the structures of bulge-containing oligoribonucleotides demonstrated that five-base bulges with a ribose unit, which is either in the apical position or located closer to the 5′ side of a bulge, adopt the same conformation in which all nucleotides are located inside the helix. Two-nucleotide bulges have a similar structure. The cleavage rates of two- and five-nucleotide bulges in which a ribonucleotide is located closer to the 5′ end are, respectively, 3 and 6 times lower than the cleavage rate of a five-nucleotide bulge with ribose in the apical position. The geometric parameters of phosphodiester bonds determined by molecular dynamics methods for all types of bulges are substantially different from the parameters typical of the in-line configuration of these bonds.[81] Presumably, the lability of phospho-

diester bonds in bulges is an important feature responsible for sensitivity of these bonds to cleavage.

The above-considered data show that most bulges in RNAs and RNA·DNA complexes can be cleaved by divalent metal ions, cleavage of bulges being different from that of mismatches and apical hairpin loops. Cleavage of bulges has a random character. Thus, phosphodiester bonds located on the 3′ side (in some cases, on the 5′ side) from bulges are cleaved. In addition, some phosphodiester bonds in an RNA·DNA duplex located near a bulge loop can undergo cleavage. In most cases, RNA cleavage at the bonds in bulges has no effect on the pattern (level) of phosphodiester bond cleavage in other RNA regions.

3. RNA cleavage within artificial bulge loops by metal complex – oligonucleotide conjugates

One of promising approaches to selective RNA injury is based on RNA cleavage by antisense oligonucleotide conjugates containing RNA-cleaving groups. First conjugates containing an RNA-cleaving group covalently bound to the 5′ end of oligonucleotides were constructed in the mid-1990s.[82, 83] These conjugates cleaved RNA targets at bonds located in a single-stranded region on the 3′ side from heteroduplexes. However, the conjugates described [82, 83] cannot cleave RNAs in a catalytic manner because of slow dissociation of complexes of the conjugate with the cleavage product. To cleave RNAs in a catalytic manner, it is necessary that an RNA·conjugate complex be more stable than the cleavage product·conjugate complex. In other words, RNA cleavage should occur within RNA·oligonucleotide duplexes. However, such duplexes are poor substrates for cleavage because the catalytic (imidazole- or metal-containing) group in such substrates occurs in the double-stranded RNA region.[84–86]

This problem can be solved by creating artificial bulges in RNAs using hybridisation with partially complementary oligonucleotides containing RNA-cleaving groups. Haner and co-workers were the first to apply this approach.[86] They used oligodeoxyribonucleotides that contained thymidine modified by a La^{3+} or Eu^{3+} macrocomplex in the middle of the sequence (Fig. 8 *a*). Upon binding to RNA, these conjugates induced the formation of two-nucleotide AA or GA bulges. In this case, a modified thymidine bearing a metal complex was located not exactly opposite to the artificial bulge in the RNA target but was shifted to the 3′ or 5′ end of RNA by 4 nucleotides, due to which the RNA-cleaving group can approach the bulged bonds from the side of either the minor or major groove of the duplex.

Conjugates of oligonucleotides entirely complementary to a 32-mer RNA target (except for two nucleotides that form a bulge in the RNA) efficiently cleave the phosphodiester bonds in this bulge and near the bulge (the extent of cleavage is 92%, 16 h), the conjugates containing the Eu^{3+} complex (see Fig. 8 *a*) performing more efficient RNA cleavage compared to conjugates containing the La^{3+} complex.

The experiments demonstrated that the most favourable position of a modified base bearing an RNA-cleaving group is such that provides cleavage through the minor groove of a heteroduplex.[86] Interestingly, the conjugate, in which the Eu^{3+} complex was covalently attached to the 5′ end of a bulge forming oligonucleotide, also efficiently cleaved RNA at the phosphodiester bonds in the bulge. It should be noted that binding of RNA to the conjugate gave rise to a free 10-mer single-stranded fragment at the 5′-end region of the conjugate, which was subjected to cleavage to an insignificant extent. The cleavage occurred primarily at the bonds in the region of the artificial bulge. The above data demonstrate that the phosphodiester bonds in bulges are more sensitive to cleavage than those in other single-stranded RNA regions. This is apparently attributed to the fact that the phosphodiester bonds in bulges assume a configuration more favourable for transesterification compared to their configuration in single-stranded RNA regions.

RNA cleavage by Eu^{3+} complex – oligodeoxyribonucleotide conjugates occurred efficiently; however, the reaction proceeded in a non-catalytic manner. The replacement of an oligodeoxyribonucleotide by 2′-methoxyethoxy derivatives

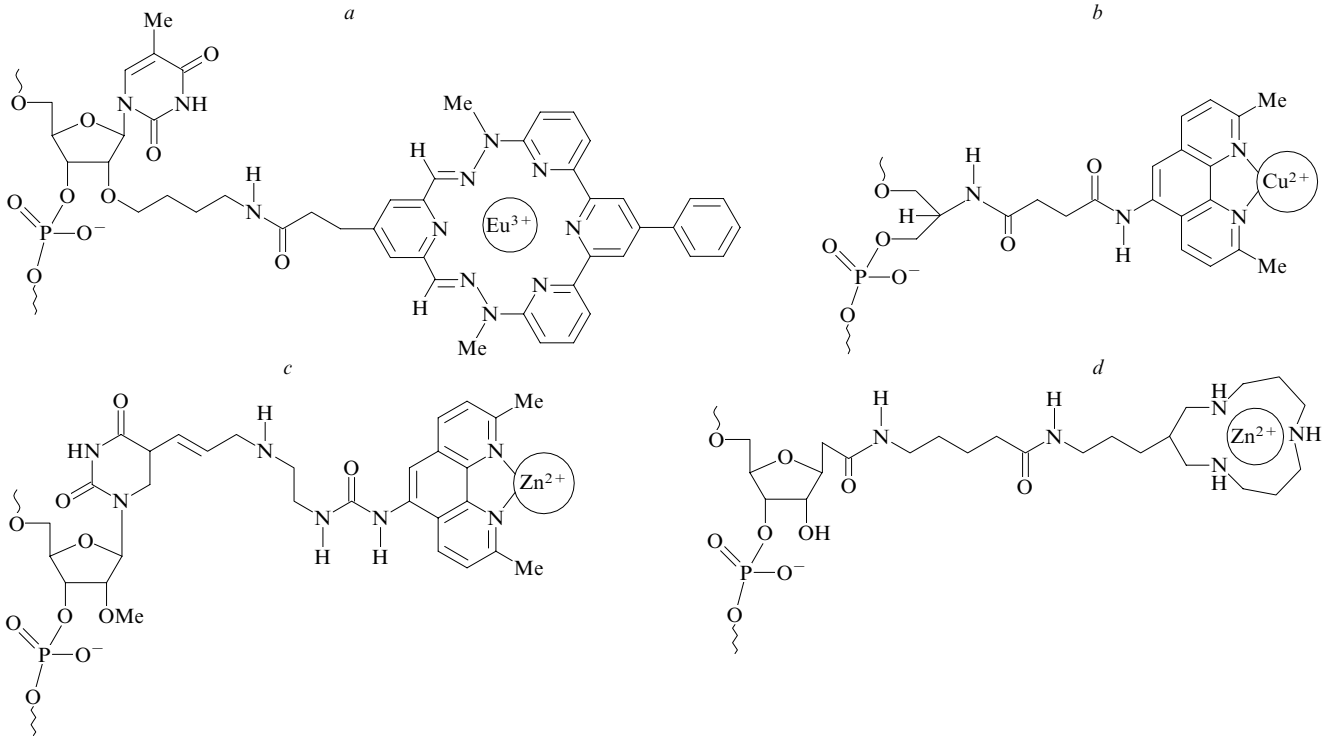


Figure 8. Structure of modified nucleotide units containing an RNA-cleaving group.[86, 89, 91, 92]

having a higher affinity for RNA enabled a decrease in the length of an antisense oligonucleotide by a factor of two.[87] The conjugate of this 10-mer oligonucleotide with the Eu^{3+} complex cleaved a 23-mer RNA target in a catalytic manner; one molecule of the conjugate was able to cleave 39 RNA molecules under physiological conditions in 64 h.[88]

Another type of RNA-cleaving conjugates includes oligonucleotides in which one nucleotide is modified by phenanthroline (Fig. 8 *b*) or terpyridine complexes of Cu^{2+} or Zn^{2+}. It was demonstrated[89] that site-directed RNA cleavage at the phosphodiester bond in an artificial single-base bulge can be performed with the use of oligonucleotide conjugates containing serinol modified by phenanthroline or terpyridine Cu^{2+} complexes instead of one of nucleotides. As opposed to conjugates described earlier,[86] metal complexes in the conjugates under consideration were attached not to ribose but to its analogue (serinol) incorporated into the ribose-phosphate backbone of an antisense oligodeoxyribonucleotide.[89] This non-nucleotide insertion was located directly opposite to artificial RNA bulge. Depending on the nature of the copper complex, the efficiency of cleavage decreased in the series: 2,9-dimethyl-*o*-phenanthroline complex $\gg$ terpyridine complex > *o*-phenanthroline complex. The extent of site-directed 159-mer RNA cleavage by the 2,9-dimethyl-*o*-phenanthroline complex was as high as 80%. Unfortunately, the authors failed to perform this reaction in a catalytic fashion. Efficient RNA cleavage was observed only in the presence of a 500-fold excess of the conjugate with respect to the RNA substrate.

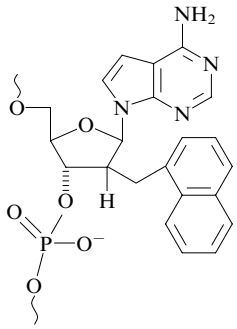
Stromberg and co-workers[90,91] synthesised 2′-*O*-methyloligoribonucleotides containing a modified uridine (Fig. 8 *c*) or cytidine residue bearing the 2,9-dimethylphenanthroline zinc complex in the middle of the nucleotide sequence. The oligoribonucleotides used were completely complementary to the RNA under study, except for nucleotides involved in homo-A bulges containing from 1 to 5 nucleotides. These conjugates cleave RNAs at the phosphodiester bonds in bulges containing from 2 to 5 nucleotides in a catalytic fashion, single-base bulges remaining uncleaved.

Unlike macrocyclic Ln^{3+} complex – oligonucleotide conjugates, which cleave RNAs at the phosphodiester bonds in bulges or near the bulges, conjugates with zinc complexes cleave RNAs only at the bonds in bulges.[90] The cleavage rate depended on the length of an artificial bulge and the mode of attachment of the 2,9-dimethylphenanthroline Zn^{2+} complex to the antisense oligonucleotide. Four-nucleotide AAAA bulges were cleaved most efficiently, the phosphodiester bonds between the first three adenosine residues were cleaved much more rapidly than the last bond in the bulge.

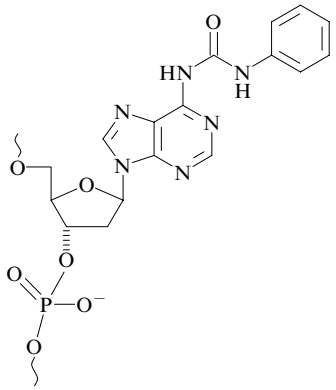
A comparison of different modes of covalent binding of the 2,9-dimethylphenanthroline zinc complex to heterocyclic bases demonstrated that the attachment at the C(5) atom of uracil is most favourable (see Fig. 8 *c*). Apparently, this arrangement of the complex allows the Zn^{2+} ion to approach the internucleotide bonds in bulges at the shortest distance. A conjugate containing two nucleotides modified by the 2,9-dimethylphenanthroline Zn^{2+} complex (in the middle and at the 5′ end of the oligonucleotide chain) was synthesised to increase the efficiency of RNA cleavage. Surprisingly, the presence of the second RNA-cleaving group led to a sharp decrease in the extent of RNA cleavage, which was attributed to steric factors.[90]

Phenanthroline zinc complex – oligonucleotide conjugates efficiently cleaved RNAs in the presence of a 4-fold excess of an RNA with respect to the conjugate. However, due to weak complexation of Zn^{2+} ions with phenanthroline, the presence of a 25-fold excess of Zn^{2+} ions in the reaction mixture is necessary for the reaction to proceed efficiently. To decrease the concentration of Zn^{2+} ions in the reaction mixture, complexes of macrocyclic polyamines, in particular, 1,5,9-triazacyclododecan-3-yl (aza crown) zinc complexes (Fig. 8 *d*),[92] were used instead of phenanthroline zinc complexes. In solution, these complexes are four times more stable than phenanthroline complexes. These conjugates also cleaved oligoribonucleotides at the phosphodiester bonds in artificial bulges, but the reaction rate was an order of magnitude lower than that achieved with conjugates containing phenanthroline complexes.

Stromberg and co-workers[93] suggested that antisense oligonucleotides, which bear not only an RNA-cleaving group, but also a group that decreases mobility of an RNA bulge, be used for RNA cleavage. It was found that oligonucleotides containing 2′-(1-naphthylmethyl)-2′-deoxytubercidin can specifically stabilise artificial bulges.



Other authors used an adenosine derivative containing a phenylurea substituent at position 6 of the heterocyclic base.[94]



This modified adenosine has a high ability of stacking interactions with adjacent bases and corresponds in size to the complementary A · T base pair. When this nucleoside is present in a duplex, it pushes the opposite nucleotide out of the helix. The phosphodiester bonds on the 3′ and 5′ sides with respect to these bulged-out nucleotides are cleaved by magnesium ions at a higher rate than the phosphodiester bonds in a single-base bulge or a mismatch. The construction of conjugates, which contain simultaneously nucleosides (adenosine) bearing groups that stabilise bulges and nucleosides bearing an RNA-cleaving group, is presently underway.

4. RNase A- and artificial RNase-induced cleavage at bulge sites

Earlier, it has been demonstrated that RNA cleavage under physiological conditions proceeds efficiently in the presence of small synthetic molecules bearing RNA-cleaving groups. These molecules mimic active sites of natural RNases.[21,22,25] Imidazole is the simplest RNA-cleaving agent. In concentrated solutions of this compound (1 or 2 M), RNA undergo random cleavage at the phosphodiester bonds located in single-stranded regions.[69] More complex RNA-cleaving compounds (artificial RNases, artificial ribonucleases; hereinafter referred to as aRNases) generally contain an RNA-binding fragment

(for example, an intercalator, a cationic structure or an oligonucleotide).[21, 22, 24, 95]

The sensitivity of RNA bulges to imidazole, RNase A and various aRNases was examined.[96, 97] A 96-mer RNA fragment of influenza virus M2 protein mRNA was used as an RNA target. In this fragment, bulges of different length (from 1 to 7 nucleotides) and sequences were formed by hybridisation with partially complementary oligodeoxyribonucleotides (Fig. 9) analogously to a procedure used earlier.[68]

It was demonstrated that the character of RNA cleavage within the bulges induced by imidazole is similar to the cleavage by metal ions, *i.e.*, all phosphodiester bonds in bulges and all bonds in a single-stranded RNA regions are cleaved. However, unlike RNA cleavage by magnesium ions, imidazole does not induce cleavage of the bonds adjacent to the bulges (<5 nucleotides). Cleavage of RNA containing longer bulges (from 5 to 7 nucleotides) by imidazole occurs at the phosphodiester bonds both in the bulges and adjacent regions. Presumably, this is associated with the fact that RNA oligonucleotide complexes containing short bulges are more stable than complexes containing longer bulges. In the latter complexes, RNA remaining in the dissociated state over a long period of time ('breathing' complexes).[96]

Natural RNase A cleaves RNAs at the phosphodiester bonds in all bulges except for single-base bulges. In addition to the phosphodiester bonds in bulges, RNase A cleaves phosphodiester bonds in other single-stranded RNA regions. Similar pattern of RNA cleavage was observed for aRNases. The extent of phosphodiester bond cleavage in bulges depends on the length and the nucleotide sequence of the bulges.

The concept 'selectivity' was introduced to estimate the difference in sensitivity of the phosphodiester bonds in artificial bulges and other phosphodiester bonds. The selectivity means the ratio of the extent of RNA cleavage at the bonds in a bulge to the total extent of RNA cleavage including all other phosphodiester bonds. It appeared that the selectivity of cleavage depends on the type of aRNase. RNase A cleaves the bonds within bulges with a low selectivity (no higher than 0.1). In other words, cleavage at the bonds in bulges occurs at a rate lower than or similar to that at the other cleaved phosphodiester bonds in RNAs.

The selectivity of RNA cleavage by artificial RNases, which are short cationic peptides, was also low.[97] The highest selectivity of cleavage was observed for aRNases containing the 1,4-diazabicyclo[2.2.2]octane residue.[98, 99] The CA phosphodiester bond in a four-membered bulge was cleaved with the highest selectivity (0.4). The cleaved phosphodiester bond was in the apical position. The presence of the second CA motif in a bulge increases the selectivity of phosphodiester bond cleavage up to 0.5.

Interestingly, the selectivity of cleavage in RNA · oligonucleotide complexes, in which the oligonucleotide contains a non-complementary adenosine residue opposite to the bulge, was always higher than that in complexes in which this non-complementary base was absent. It should be noted that, according to the data from gel shift analysis, binding of oligonucleotides containing an additional adenosine residue to RNAs does not differ from binding of oligonucleotides in which a non-complementary adenosine residue is absent.[96] Consequently, in this case high selectivity of cleavage can be attributed to a more flexible structure of the bulge, which provides for efficiency of transesterification. Therefore, artificial ribonucleases containing the 1,4-diazabicyclo[2.2.2]octane residue, unlike short cationic peptides, predominantly cleave bonds in bulges.

5. RNA cleavage at bulge sites by artificial RNases in the presence of magnesium ions

It is known that Mg^{2+} ions stabilise the RNA structure by decreasing its mobility,[100] resulting in a decrease in efficiency

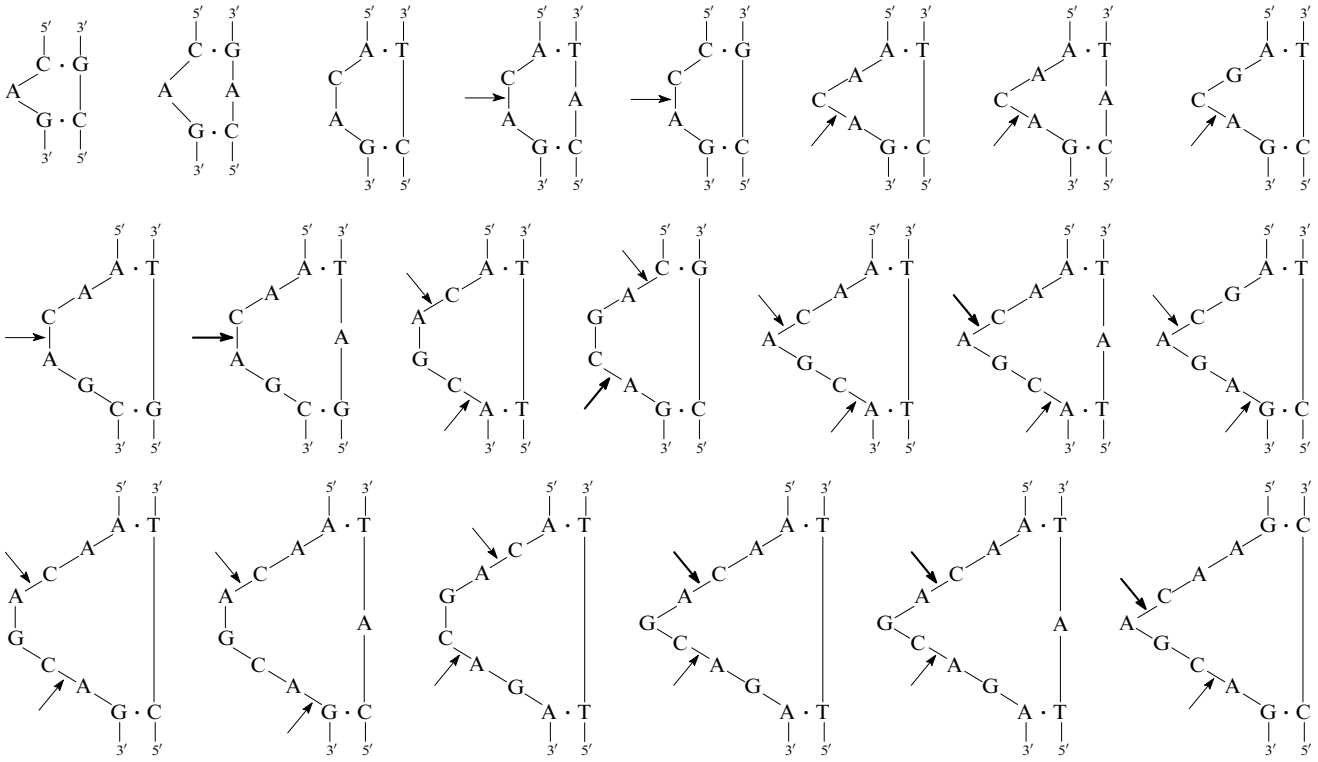


Figure 9. Sensitivity of the phosphodiester bonds in RNA bulges to cleavage by artificial RNases (the arrow thickness corresponds to the cleavage efficiency of a particular phosphodiester bond).[96, 97]

of RNA cleavage by different agents. At the same time, magnesium ions stabilise such RNA structural elements as bulges. In the presence of magnesium ions, the total extent of M2-96 RNA cleavage by artificial RNases decreases. However, in the presence of $MgCl_2$ at a concentration of 10 mmol litre^{-1}, RNA·oligonucleotide complexes are cleaved by aRNases containing 1,4-diazabicyclo[2.2.2]octane predominantly at the bonds in bulges, whereas cleavage at other regions is suppressed to a substantial degree.[96,97] In the presence of magnesium ions, the selectivity of cleavage of four- and seven-membered bulges increases to 0.68 and 0.75, respectively, *i.e.*, the formation of bulges in RNAs entirely changes the cleavage pattern by aRNases containing 1,4-diazabicyclo[2.2.2]octane. An increase in selectivity of cleavage in the presence of magnesium ions was observed for neither RNase A nor short cationic peptides.[97]

These data correlate with the X-ray diffraction data for the single-nucleotide A bulge in the [r(GCG)d(ATATA)r(CGC)]$_2$ duplex. In the presence of Mg^{2+} ions, the phosphodiester bond in the bulge adopts a nearly in-line configuration, unlike the configuration observed in the crystals grown in the presence of spermine.[41] Apparently, Mg^{2+} ions can stabilise the in-line configuration of the phosphodiester bonds in bulges, which is favourable for transesterification.

IV. Conclusion

The idea for construction of specific catalysts for RNA cleavage has attracted attention for many years. Theoretical studies demonstrated that oligonucleotides that contain RNA-cleaving groups and form artificial bulges in a complementary RNA sequence can selectively inactivate RNAs in a catalytic manner and do not require additional enzymes or cofactors. However, there are problems with implementation of this approach in practice. The sensitivity of the phosphodiester bonds in bulges depends not only on the length and the nucleotide sequence of the bulge, but also on the nature of the RNA-cleaving group. An RNA-cleaving group should not only be an efficient catalyst for cleavage, but also be in an optimal orientation with respect to the cleaved bond. One of requirements imposed on the oligonucleotide moiety of the conjugate is that the DNA·RNA complex should be sufficiently stable before cleavage and readily dissociate after cleavage. In the case of long RNAs, this problem is complicated by a search for RNA regions accessible for binding with oligonucleotides. Therefore, the construction of efficient artificial RNases performing site-directed RNA cleavage requires a complex approach.

Two main approaches are available for site-directed RNA cleavage by artificial RNases. One approach is based on the synthesis of antisense oligonucleotides containing RNA-cleaving groups (see Section III.3). Another approach consists in constructing binary systems composed of a low-molecular-weight RNA-cleaving unit and a complementary oligonucleotide. The latter contains a group enhancing the sensitivity of a particular region or particular bonds in RNAs to cleavage.[96,97,101] Site-directed RNA cleavage with the use of this system was first exemplified by cleavage of a short model 36-mer RNA in which all bonds, except for the bond subjected to cleavage, were protected by complexation with complementary oligonucleotides.[93]

The approach proposed be Vlassov *et al.*[96] is based on an increase of sensitivity of phosphodiester bonds in RNAs to cleavage due to their bulging. It was demonstrated that in the presence of magnesium ions, the phosphodiester bonds in (4–7)-member bulges, which are formed in RNAs by hybridisation with a partially complementary oligonucleotide, are selectively cleaved by aRNases at a rate several times higher than the rate of cleavage of other phosphodiester bonds in RNA molecules. Selective RNA cleavage within a bulge occurs apparently due to the appearance of constrains, which is necessary for efficient transesterification, and stabilisation of the other parts of the RNA molecule in the presence of magnesium ions. As a result, the RNA molecule becomes poorly sensitive to cleavage at all bonds except those in bulges.[96,97] To summarise, selective RNA cleavage at phosphodiester bonds in bulges can be performed with the use of a binary system consisting of aRNase and an oligonucleotide partially complementary to RNA.

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