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Design of site-specific RNA-cleaving reagents

V N Silnikov, V V Vlassov

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Abstract. Data on chemical compounds capable of cleaving ribonucleic acids under physiological conditions are systematised and generalised. The possibilities of the design of site-specific RNA-cleaving reagents are considered. The bibliography includes 245 references.

I. Introduction

Reagents capable of performing efficient and specific cleavage of nucleic acids (NA) are required in molecular-biological investigations, used for the development of genetic-engineering techniques and employed in genetic engineering and gene therapy. Selective NA-specific cleaving reagents can be used for the preparation of biologically active gene-targeting compounds capable of suppressing or modulating expression of particular genetic programs. The design of such compounds offers possibilities of influencing the origin of pathological states.[1–4] This line of investigation can revolutionise the strategy of treatment of virtually all diseases.

The construction of NA-cleaving reagents for all the above-mentioned applications is a complicated problem because cleavage should proceed under nearly physiological conditions. Preparations used as therapeutic drugs should act strictly selectively on particular sequences in the compositions of certain types of nucleic acids. Side processes must be excluded, and the efficiency of the reaction with the target nucleic acid should be high to suppress functioning of the latter as completely as possible.

Since the design of gene-targeting chemical reagents is a topical problem, this line of investigation has been developed intensively in recent years. Presently, the following types of compounds are primarily used for DNA cleavage.

1. Compounds containing variable-valence metal complexes as reactive groups. The latter catalyse oxidation processes resulting in nucleoside degradation.[5–7]

2. Enediynes capable, under certain conditions, of generating biradical species, which react with both DNA chains and cause their cleavage.[8]

3. Alkylating reagents whose reactions weaken the glycoside bond in modified nucleosides resulting in their degradation.[9,10]

Recently, reagents cleaving DNA according to a photooxidation mechanism[11,12] as well as reagents based on natural and synthetic metal-independent antibiotics (varacin, lissoclinotoxin, *etc*.) generating hydroxyl radicals[13] have gained wide acceptance. Mechanisms of DNA hydrolysis, which occurs through interactions of radicals with various nucleic-acid elements, were considered in detail in reviews.[5,14,15]

Unlike DNA, RNA are substantially more stable to oxidative cleavage[16] (in particular, under the action of metal-dependent antibiotics, such as bleomycin,[17,18] and enediyne antibiotics, such as neocarzinostatin, esperamycin, *etc*.[19]) due to higher stability of the glycoside bond in ribonucleotides compared to that in deoxyribonucleotides. At the same time, hydrolytic cleavage of RNA proceeds much more readily.

Recently, considerable study has been given to the mechanisms of RNA cleavage with various compounds and to the design of catalysts for directed RNA cleavage. Data accumulated in this field and the construction of the first chemical RNA-cleaving agents exhibiting higher specificity compared to that of natural enzymes call for generalisation and analysis of the results of the investigations.

A very interesting line of investigation aimed at designing RNA-targeting agents involves the construction of ribozymes, *viz*., catalysts based on RNA or DNA. Different classes of ribosimes, procedures for their construction and the mechanisms of RNA cleavage under the action of these catalysts were considered in detail in reviews.[20–24]

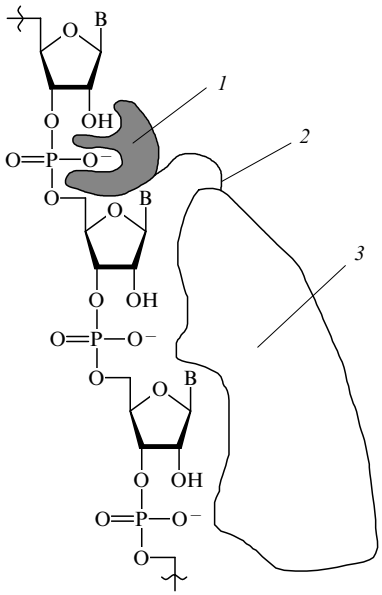
The results of studies in the field of construction of artificial catalysts whose active groups catalysing hydrolysis of phosphodiester bonds are involved in conjugates with oligonucleotides, peptides and intercalates have not been systematised. The present review is intended to close this gap and is devoted to the main classes of compounds capable of catalysing RNA depolymerisation under physiological conditions and to their use in the preparation of 'synthetic ribonucleases' for directed cleavage of particular sequences of ribonucleic acids.

II. General problems of the design of site-specific RNA-cleaving reagents

Compounds capable of performing selective site-specific RNA cleavage must, like enzymes, contain two domains, *viz*., an RNA-binding domain, which can form a specific complex with particular RNA regions (target regions), and an RNA-hydrolysing domain performing hydrolysis of phosphodiester bonds adjacent to the binding sites or located in their vicinity (Fig. 1). Some

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B, heterocyclic base residue.

Figure 1. Overall structure of synthetic ribonucleases;
(*1*) an RNA-hydrolysing fragment or a reactive group, (*2*) a linker, (*3*) an RNA-binding fragment.

peptides forming complexes with particular nucleotide sequences or elements of the three-dimensional RNA structure as well as low-molecular-weight organic compounds, such as intercalators,[25] polycations [26] and aminoglycosides,[27] can provide selectivity of RNA binding. Selected aspects of interactions of low-molecular-weight ligands with nucleic acids have been considered previously.[28–30] Oligonucleotides and their analogues are the most promising affine structures for directed action on RNA. A complementary oligonucleotide can be directed at any nucleotide sequence of interest. An additional advantage of these reagents is the fact that all these compounds can be synthesised with the use of a universal technology. Nucleotide sequences consisting of 15–20 units are unique even in genomes with complex organisation, this length of complementary oligonucleotides being sufficient to provide recognition and efficient binding with an RNA target under physiological conditions. Recently, a series of oligonucleotide analogues, which are resistant to cell enzymes and can form more stable complexes with nucleic acids than those of natural oligonucleotides, have been designed.[31–36]

Natural RNA-hydrolysing compounds, *viz*., enzymes and antibiotics, can be used as RNA-cleaving groups. Thus compounds, which can perform selective RNA cleavage in the vicinity of sequences involved in binding of conjugates, were synthesised based on oligodeoxyribonucleotides and natural enzymes, *viz*., ribonucleases A, S [37,38] and H.[39,40] However, enzymes contain their own RNA(DNA)-binding affine structures along with catalytic centres, which is necessarily reflected in the specificity of such conjugates and in the effects caused by these conjugates in biological systems. Due to this fact as well as to the complexity of the synthesis of these conjugates, enzymes and natural antibiotics show little promise as reactive groups.

RNA-hydrolysing fragments or reactive groups whose properties have little effect on the recognition of nucleic acid by a conjugate are agents of choice.

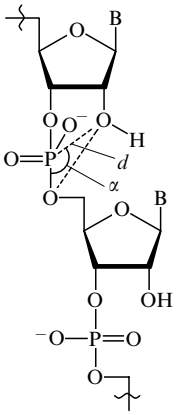
III. Catalysts of RNA cleavage

The presence of 2′-hydroxyl groups in RNA makes possible hydrolytic cleavage of these biopolymers according to the mechanism of intramolecular transesterification. RNA cleavage by this mechanism is widespread in nature, for example, in reactions catalysed by ribonucleases and ribozymes. It is reasonable to employ processes occurring in active centres of ribonucleases and ribozymes in the construction of RNA-cleaving chemical reagents.

It should be taken into account that high efficiency of natural enzymes results from functioning of complex active centres that arise in the course of evolution, while reactive groups forming a catalytic centre taken alone are generally weak catalysts of the corresponding processes. In the construction of artificial catalysts for the action on biopolymers, it is very important to provide the necessary spatial arrangement of all participants of the catalytic process by using the corresponding linkers.

1. Mechanism of hydrolytic cleavage of ribonucleic acids

Many enzymes and ribozymes cleave ribonucleic acids according to the mechanism of intramolecular transesterification through the nucleophilic attack of the oxygen atom of the 2′-hydroxyl group at the phosphorus atom. This reaction can proceed by two stereochemical mechanisms. One mechanism is realised when the angle α (Fig. 2) is close to 180° and the distance d between the



B, heterocyclic base residue.

Figure 2. Principal parameters responsible for the reactivity of phosphodiester bonds in RNA;
d is the 2′-O–P distance (Å) and α is the 5′-O–P–3′-O angle.

reacting groups < 3 Å (the linear mechanism), whereas another mechanism takes place when the angle α is close to 90° (the related mechanism). In the former case, the AE mechanism (an analogue of the S_N2 mechanism) is realised. The latter mechansim requires an additional stage, *viz*., migration of the leaving group from the equatorial to apical position (pseudorotation).[41] Pseudorotation was postulated for hydrolysis of a number of model compounds.[42–44] It was assumed that 3′→ 2′-isomerization in ribonucleotides also involves pseudorotation.[45] However, hydrolysis proceeded according to the linear mechanism in all cases for which the mechanism of catalytic RNA cleavage under the action of ribozymes or enzymes was established. Possible mechanisms of hydrolytic RNA cleavage under various conditions were considered in detail in a number of reviews and original studies.[45–47] The structural parameters of the regions of some RNA in which spontaneous hydrolysis is highly probable were analysed.[48] The dimensionless parameter F varying from 0 to 1 (for notations of α and d, see Fig. 2) was used as an integral value characterising stability of phosphodiester bonds in RNA sequences.

$$F = \frac{\alpha - 45}{180 - 45} \frac{(3\text{Å})^3}{d^3}.$$

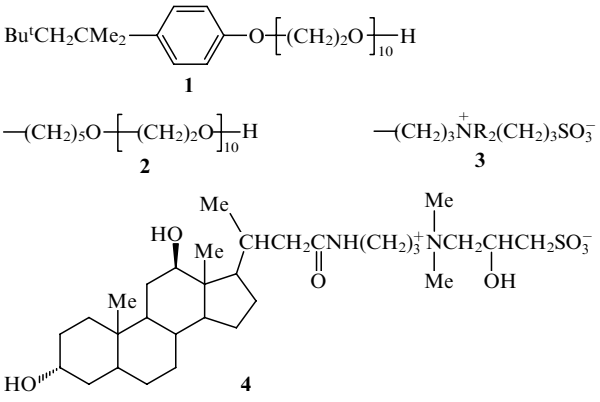
It appeared that the parameter F characterising the mutual spatial arrangement of the oxygen atom of the 2′-hydroxyl group, the phosphorus atom and the oxygen atom at the 5′-terminus of

the leaving nucleoside correlates well with stability of the internucleotide bonds. The structural elements with $F = 0.5-0.8$ correspond to sites of spontaneous hydrolysis, whereas stable regions are characterised by the parameter F of 0.1–0.15. Hydrolytically labile sites with low values of the parameter F are rare exceptions. Generally, heterocyclic bases in these sites form either noncanonical GA pairs or CpA(UpA) sequences in single-stranded regions in which short-lived structures (which are able to undergo cleavage according to the linear mechanism, but which are not detected by NMR spectroscopy and X-ray diffraction analysis) can, apparently, arise.

Low values of the parameter F are also typical of double-stranded RNA regions in which the phosphodiester bonds are characterised by high stability.

It should be noted that experimental data on the high hydrolytic lability of some sequences in RNA are available. However, no explanation for these data was offered. Thus, cleavage of the phosphodiester bonds in the CpA(UpA) sequences in single-stranded RNA occurs most readily, which is not clearly understood.[49] These sequences in short single-stranded oligoribonucleotides undergo cleavage in the presence of various proteins (T7 RNA polymerase, T4 polynucleotide kinase, lysozyme or trypsin) and some polymers, such as polyethylene glycol, dextran or polyvinyl pyrrolidone.[50–52] The efficiency of cleavage depends on the length of the oligonucleotide and on the nature of the flanking nucleotides. Thus, the ratio between the rates of hydrolysis of the bonds in the UA sequence for GUAC, GUAU and CGUA is 1 : 6 : 16, whereas the corresponding ratio for GUAA, CGUAA and UCGUAA is 1 : 4 : 6. The change of the nucleotide residue X at the 3′-terminus in the XUAA tetranucleotide has virtually no effect on the rate of hydrolysis, whereas the efficiency of GUAX cleavage decreases in the series X = G > A > U > C by a factor of three.[53]

It was also demonstrated that the UpA and CpA sequences in the loop regions of RNA are cleaved in the presence of nonionic [Triton X-100 (**1**) or Genapol X-100 (**2**)] or zwitterionic [zwittergent 3–10 (**3**) or an amide derivative of cholic acid (**4**)] detergents at concentrations corresponding to the critical concentrations of micelle formation.[54] Efficiency of hydrolysis is independent of the micelle size. The maximum rate of hydrolysis is achieved in the case of the loop consisting of five nucleotide units.[50] Anionic detergents, such as sodium dodecyl sulfate or deoxycholic acid, suppress cleavage.

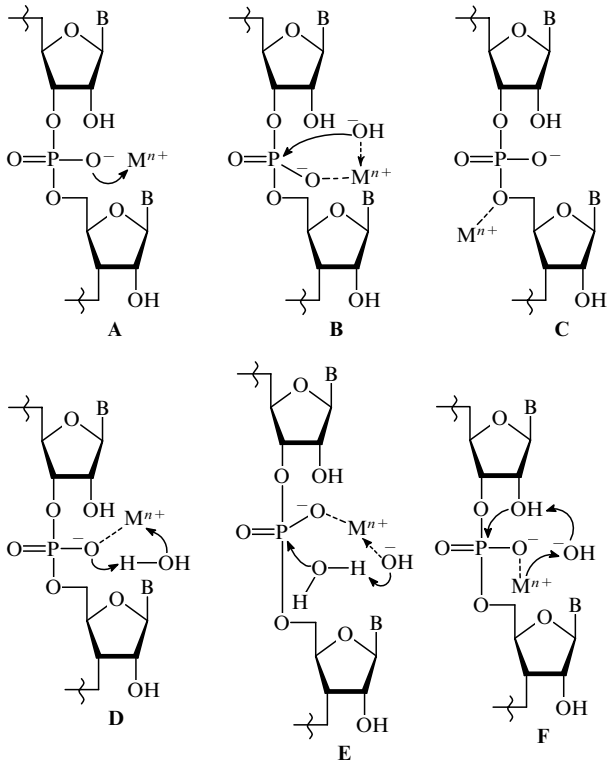


It can be suggested that the compounds **1**–**4** influence RNA in such a way that in some of its regions, *viz*., in the pyrimidine–purine sequences, the oxygen atom of the 2′-hydroxyl group of ribose and the internucleotide phosphate are brought into proximity and the angle α is increased, the resulting geometry being most favourable for spontaneous cleavage of the phosphodiester bonds. However, the mechanism of this reaction as well as the reasons for high lability of the CpA and UpA fragments still remain to be elucidated.

2. Hydrolysis of ribonucleic acids involving metal ions and metal complexes

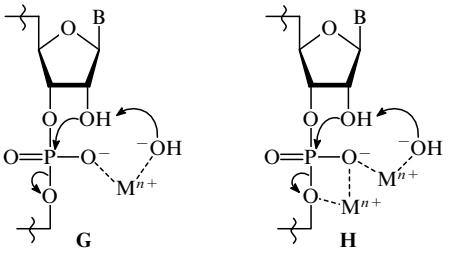
Presently, abundant data on hydrolysis of ribonucleic acids involving various metal ions are available. Destruction of ribonucleic acids under the action of the Zn^{2+}, Ni^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Al^{3+} and Ln^{3+} ions and ions of other lanthanides has received the most study. Some of these ions can cause RNA cleavage under physiological conditions.

Metal ions are involved in active centres of many enzymes hydrolysing phosphodiester bonds, such as phosphomono-,[55] phosphodi-[56,57] and phosphotriesterases.[58] In some cases, these ions serve as cofactors necessary for functioning of ribozymes taking part in chemical catalysis of cleavage of phosphodiester bonds.[59] Below are considered the schemes of cleavage of phosphodiester bonds in RNA with the participation of metal ions. Metal ions can be involved in catalytic processes as Lewis acids by reacting directly with the oxygen atom of the phosphate group (**A**),[60] with a nucleophile (for example, with the hydroxide anion) (**B**)[61] or with the oxygen atom of the leaving group (**C**). In addition, metal ions can be indirectly involved in catalysis. Thus, these ions can either coordinate a water molecule and act as intramolecular general acid catalysts (**D**) or coordinate the hydroxide anion and act as intramolecular general base catalysts (**E** or **F**).[62,63]



Apparently, hydrolytic RNA cleavage with different metal ions can proceed by one or another mechanism or according to a combination of them. Two main models of the participation of metal ions in hydrolysis of phosphodiester bonds were proposed. According to the model **G**, the metal ion and the hydroxide anion coordinated to this ion are involved in the catalytic reaction.[64–68] The model **H** is based on the mechanism accepted for such natural enzymes as alkaline phosphatase and DNA polymerase I[57,69] and assumes the participation of two metal ions one of which coordinates the hydroxyl group and the second ion activates the oxygen atom of the leaving group. Most of the results obtained in studies of RNA hydrolysis catalysed by metal ions can be interpreted based on both the models **G** and **H**.[70] X-Ray diffraction data for some ribozymes provide evidence in favour of the reaction mechanism involving one metal ion.[64,71] However, information on substantial acceleration of RNA hydrolysis

under the action of the $Mg^{2+}-Pb^{2+}$ (see Ref. 72), $Nd^{3+}-Pb^{2+}$ (see Refs 73 and 74), $La^{3+}-Fe^{3+}$ and $La^{3+}-Sn^{4+}$ pairs [75] can be interpreted only within the framework of the model **H**.



G **H**

Using the ApA dinucleotide as an example, it was demonstrated that the phosphodiester bond can be hydrolysed under the action of both the hydroxy complex of La^{3+} (see Ref. 76) and the binuclear hydroxy complex $[La_2(OH)]_5^+$.[77] The rate of hydrolysis was increased by four orders of magnitude on going from the mono- to the binuclear complex.[78] It can be suggested that hydrolysis can proceed involving either one or two metal ions depending on the reaction conditions. Within the frameworks of all mechanisms proposed, metal ions, which are stronger Lewis acids and have a larger coordination sphere, should be more efficient catalysts of hydrolytic cleavage. In this connection, considerable study should be given to cleavage of phosphodiester bonds under the action of the Ce^{4+} (see Ref. 79) and Zr^{4+} ions.[80, 81]

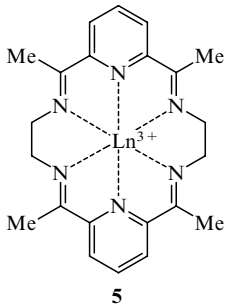
The rate of hydrolysis of phosphodiester bonds in RNA catalysed by metal ions depends on the nature of the flanking nucleosides. Thus, hydrolysis of the phosphodiester bond in ApA in a solution of the Tm^{3+} salt (0.01 mol litre^{-1}) proceeded 4.8–5.1 times faster than that in UpU.[82] In studies of hydrolysis of six different nucleotide pairs (UpG, CpC, GpA, ApU, UpA and ApA) in solutions of the Lu^{3+} salt (0.01 mol litre^{-1}), the reaction rate for the UpG dinucleotide, which underwent hydrolysis most rapidly, was found to be 2.2 times higher than that for ApA, which is the most stable under these conditions.[82] The presence of the 3′-terminal phosphate group leads to substantial acceleration of hydrolysis of the internucleotide phosphate bond in dinucleotides due, apparently, to more efficient cation binding. Thus, hydrolysis of the phosphodiester bond in ApAp in the presence of Zn^{2+} ions proceeded more rapidly than that in ApA. At the same time, the presence of the 2′-terminal phosphate group or the 2′,3′-cyclophosphate group exerts only a slight effect on the rate of hydrolysis.[83] Analogous results were obtained in other studies as well.[84, 85] It was shown that the 3′-terminal phosphate group in oligodeoxynucleotides containing a ribonucleotide unit, *viz.*, Up(Tp)$_n$Tp or Up(Tp)$_n$T ($n = 0-4$ or 7), at the 5′-terminus accelerates hydrolysis even if it is spaced at seven nucleotide units.[86] Apparently, the observed 5–20-fold acceleration of hydrolysis of poly(U) compared to UpU under the action of the Mg^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+} or Zn^{2+} ions is attributable to the polyelectrolyte effect of the total negative charge of poly(U).[87]

The ability of metal ions to catalyse hydrolysis varies over a wide range depending on the nature of the substrate. Thus, the activity of metal ions in the hydrolysis of ApA decreases in the series Tm = Lu > Y > Nd = Eu = Sm > Ce = Sc = Gd = Tb > Pr = Dy > Ho = Er > Yb > La.[76] At the same time, the activity of metal ions in hydrolysis of 3′,5′-cycloadenosinemonophosphate (which is one of the intermediate hydrolysis products of ApA) under analogous conditions decreases in the series Ce ≫ Pr > Nd = La > Y = Sm = Dy > other lanthanides.[88]

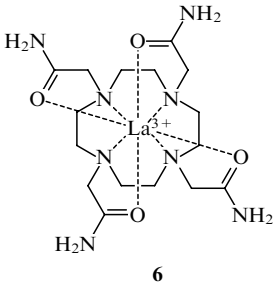
Evidently, the differences in the rate of hydrolysis in the dunucleotide pairs as well as in more complex substantces arise from the different complex-forming ability of various fragments of nucleic acids. Problems of complex formation of metal ions with nucleic acids were considered in detail in a review.[89]

Hydrolysis of RNA in the presence of various metal ions is widely used for revealing the cation-binding sites in nucleic acids.[90–94] The magnesium, lead and lanthanide ions can catalyse selective cleavage of phosphodiester bonds in the regions of some specific elements of the three-dimensional RNA structure.[95–99] The sensitivity of these reactions to the orientation of the ligands was used in studies of the RNA structure in solution and for comparison of the three-dimensional structures of mutant tRNA and 5SRNA.[100, 101] It was demonstrated [102] that sequences of ribonucleic acids in complexes with oligodeoxynucleotides do not undergo hydrolysis in the presence of Eu^{3+} or La^{3+} complexes, apparently, because the conformational flexibility of RNA, which is necessary for efficient ion binding and for transesterification, is limited upon formation of the duplex. In imperfect complexes, loop single-stranded regions are cleaved more rapidly.[103] The effects of the nucleotide sequence and the size of the loop region on the efficiency of RNA hydrolysis in the presence of the Pb^{2+} and Zn^{2+} ions as well as in the presence of their complexes were examined in detail.[104] The efficiency of hydrolytic cleavage was demonstrated to be increased as the size of the loop increases and as the hydrolysed phosphodiester bond moves away from the stalk fragment. The results obtained confirmed once again the necessity of conformational flexibility of the ribosephosphate fragment subjected to hydrolysis.

Due to the ability of complexes of lanthanides,[105, 106] copper,[107] zinc and some other metals [108] to catalyse hydrolysis of phosphodiester bonds, they can be considered as potential hydrolytically active groups in the synthesis of conjugates with organic compounds and oligonucleotides. The lanthanide ions, which are strong Lewis acids [109] and have a flexible coordination sphere,[110] are believed to be the most promising catalysts. The construction of highly stable metal complexes, which are simultaneously characterised by the high acidity of the central atom, presents a contradictory problem. However, a number of metal complexes (for example, compounds **5**–**8**) possessing high stability with retention of high activity in hydrolysis were prepared.[85, 106, 111, 112] It was demonstrated that catalytic RNA cleavage can proceed in the presence of zinc(II) [85] and lanthanum(III) [106] complexes. The rate of RNA hydrolysis in the presence of different metal complexes depends on pH. This suggests that, as in the case of free metal ions, hydrolysis of phosphodiester bonds occurs, apparently, with the participation of two molecules of the metal complex. In this case, one molecule acts as a Lewis acid, whereas the second molecule coordinates the hydroxide anion and acts as a base.[113, 114]

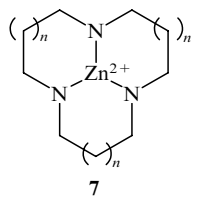


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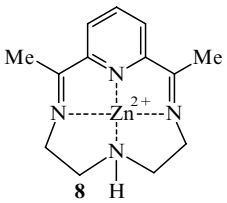


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Ln = La, Eu, Tb, Lu.



7



8

$n = 2, 3$.

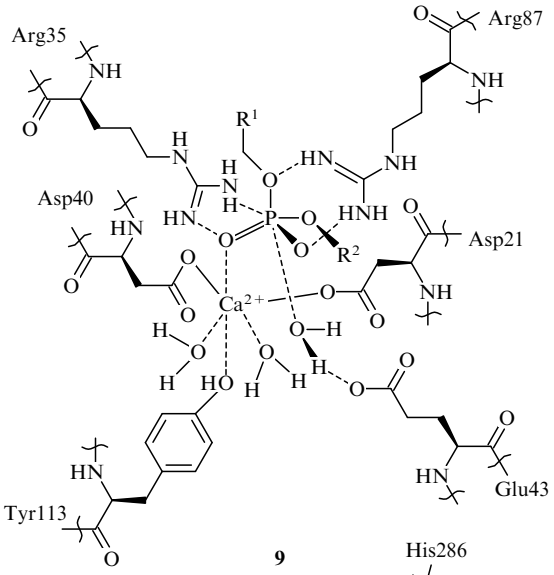
3. Reagents modelling active centres of metal-dependent enzymes catalysing phosphoester-bond cleavage

Hydrolytic centres of metal-dependent enzymes, which are involved in hydrolysis of phosphoester bonds in various sub-

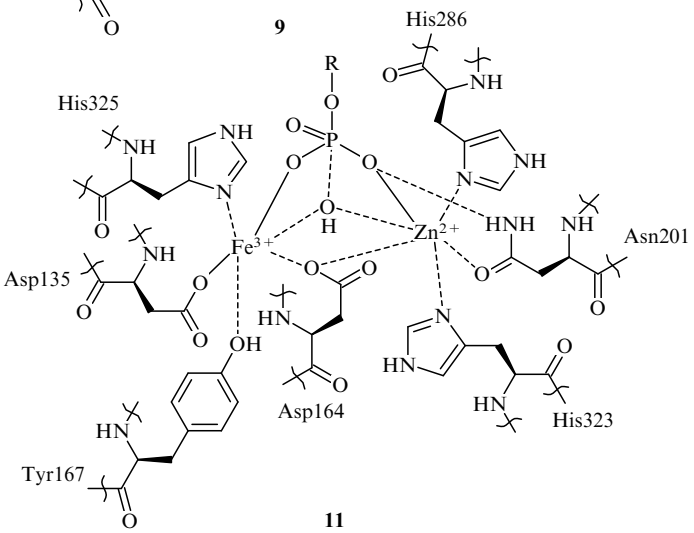
strates, often contain two complex-forming domains consisting of the amino acid residues His, Asp(Asn), Glu, Arg and Lys (in different combinations) and one of hydroxyl-containing amino acids, *viz*., Ser or Tyr. The distances between the metal ions (Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+} and Fe^{3+}) in the active centres are in the range of 3–5 Å. In addition, the hydrolytic centres may contain one or several such amino acids as Glu, Arg, Lys, His or Ser. The functional groups of these amino acids are not directly bound to the metal ions and act as additional base, acid or nucleophilic catalysts (see reviews [115–118]). Active centres of some enzymes contain one (staphylococcus nuclease [119] and nuclease Sm from *Serratia marcescens* [120]) or more than two metal ions (nuclease P1).[121] Below are considered the principal structural elements of the active centres of natural metal-dependent enzymes, which hydrolyse phosphoester bonds in various substrates and contain from one to three metal ions, *viz*., staphylococcus nuclease (**9**), phosphorylase C from *Bacillus cereus* (**10**) and purpuric acid phosphatase (**11**).

Owing to success achieved in the synthesis of stable metal complexes and progress made in the determination of the structures of active centres and elucidation of the mechanisms of action of enzymes, which hydrolyse phosphoester bonds in various substrates, a broad range of synthetic catalysts, which serve as functional and structural models of natural enzymes, have been synthesised in recent years.[122–124]

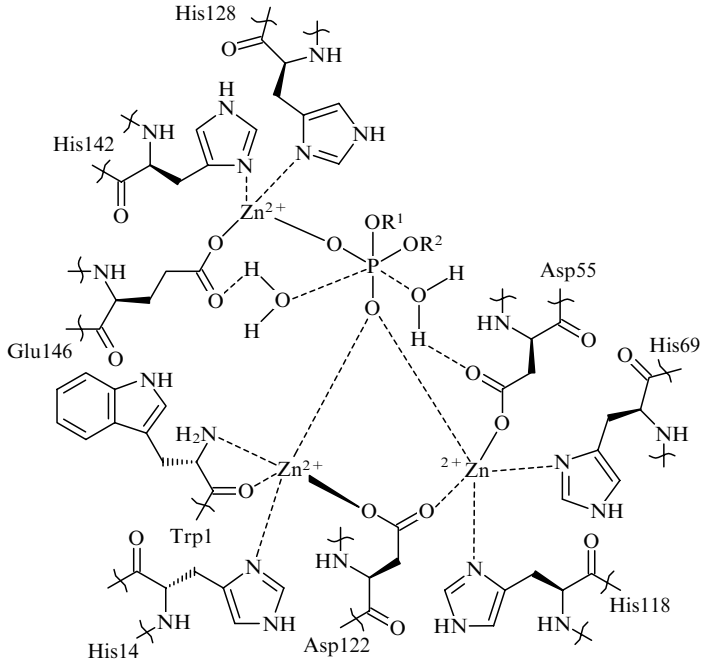
Binuclear metal complexes based on the Zn^{2+} (see Refs 125–127), Cu^{2+} (see Refs 128–130), Co^{3+} (see Refs 131 and 132) and La^{3+} ions [133, 134] were prepared. The efficiency of hydrolysis in the presence of binuclear metal complexes appeared to be much higher than that in the case of their mononuclear analogues. This is particularly essential for the Zn ions, which are weak Lewis acids and whose mononuclear complexes do not catalyse hydrolysis.[129, 135] Compounds **12**–**16** proved to be the most efficient catalysts among all polynuclear metal complexes modelling active centres of natural enzymes. In particular, hydrolysis of the phosphoester bond in the model substrate in the presence of the complex **12**, which is a structural and functional model of D-fructose 1,6-bis(phosphate-1) phosphatase, proceeded 10^3 times more rapidly than that in the presence of similar cobalt complexes.[132] Analogous results were obtained for the copper complex **13**. In the presence of the latter the rates of hydrolysis of phosphoester bonds in various compounds are increased by a factor of $(1-2.7)\times10^4$.[129] It should be noted that in the case of hydrolysis of natural substrates in the presence of polynuclear metal complexes, metal ions can interact not only with phosphate residues, but also with heterocyclic bases as a result of which these compounds acquire particular substrate specificity. Thus, the relative rates of cleavage upon hydrolysis of ribonucleoside-2′,3′-cyclophosphates of adenosine, guanine, uridine and cytosine in the presence of the binuclear copper complex **15** are 1.5, 8.5, 4.3 and 1, respectively, whereas the relative rates in the presence of the complex **16** are 31.1, 1, 2.9 and 5.1, respectively.[130] The ratio between the rates of hydrolytic cleavage of the GpG dinucleotide, which is most readily hydrolysed, and the most stable ApA dinucleotide in the presence of the trinuclear zinc complex **14** was 163 : 1.[136]



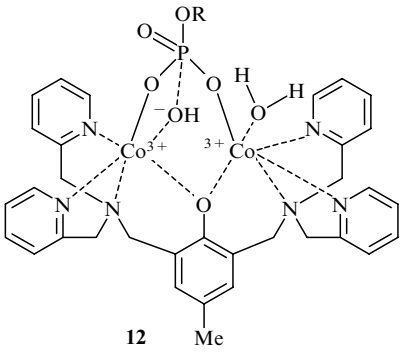
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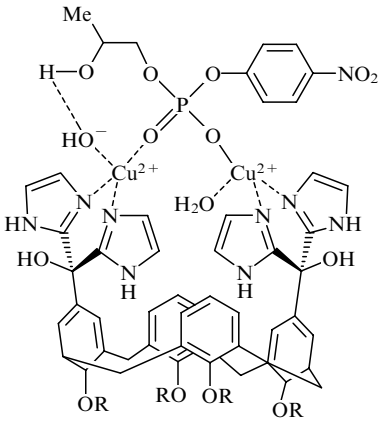


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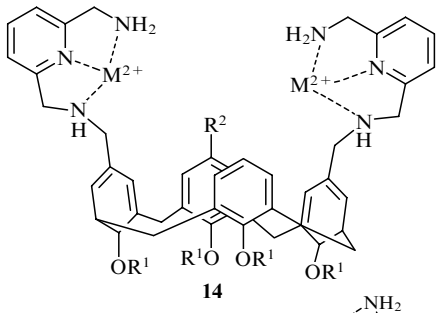


12

R = 4-$NO_2C_6H_4$.

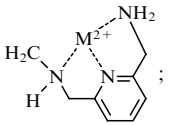


13 (R = $(CH_2)_2OEt$)

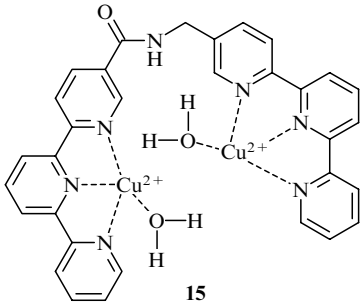


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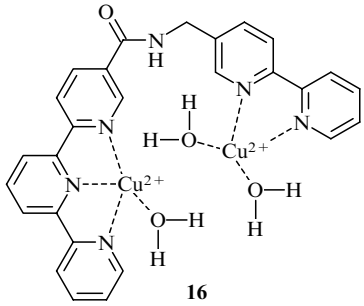
R^1 = $(CH_2)_2OEt$; R^2 = H, M = Zn, Cu.



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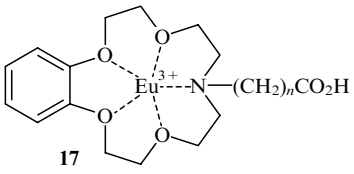


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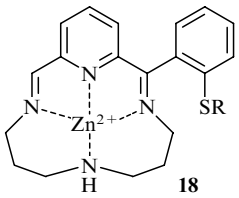
16

Below are listed compounds **17**–**22** modelling hydrolytic centres of enzymes, which act as acid catalysts (**20**–**22**), base catalysts (**17**, **18** and **22**) or nucleophilic catalysts (**19**) and whose active centres contain functional groups that are not bound to metal ions.[137–140]



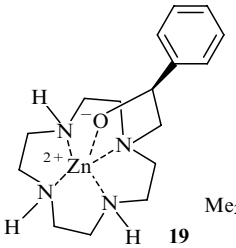
17

n = 1–3.

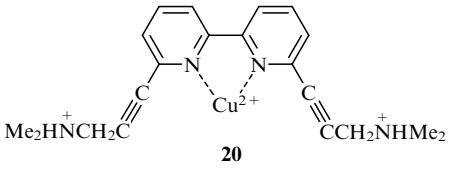


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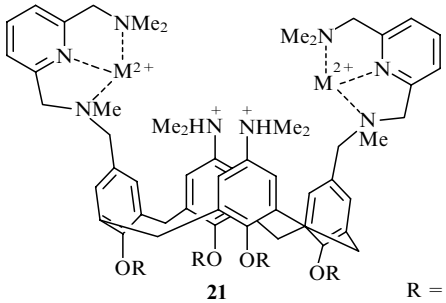
R = H, 



19



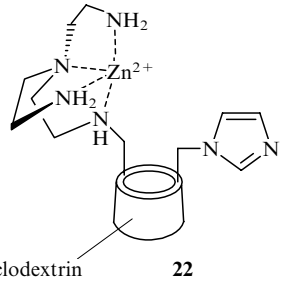
20



21

R = $(CH_2)_2OEt$

Modelling of the active centres of such enzymes requires a more rigid mutual spatial arrangement of the reactive groups compared to that in compounds containing only metal ions with flexible and bulky coordination spheres. In most cases, the compounds **17**–**22** proved to be only insignificantly more efficient than the basis metal complexes.



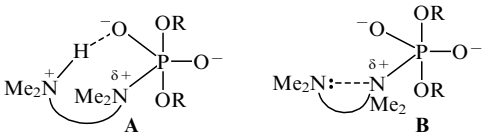
Cyclodextrin **22**

Probably, this is attributable to the unfavourable spatial arrangement of the reactive centres. The efficiency of cleavage is increased by a factor of 10^3–10^4 when the mutual arrangement of all participants of the catalytic process is optimum.[141, 142] Models of the active centres of natural enzymes based on metal complexes containing additional functional groups, which are not involved in complex formation, were discussed in detail in a review.[143]

4. Hydrolysis of ribonucleic acids with amino compounds and cationic peptides

Hydrolysis of RNA is catalysed by various amino compounds.[144] In the studies of hydrolysis of bis(4-nitrophenyl) and bis(2,4-dinitrophenyl) phosphates with diamines of the general formula $Me_2N(CH_2)_nNMe_2$ (**23a**–**c**) [n = 1 (**a**), 2 (**b**) or 3 (**c**)], two possible mechanisms of cleavage of the phosphodiester bonds were proposed. One of these mechanisms (**A**) is realised in the case of hydrolysis involving diamines as the corresponding monocations. The second mechanism (**B**) assumes the participation of neutral diamines. Thus hydrolysis in the presence of the diamines **23a**–**c** as monocations was accelerated by a factor of 870, 74 and 190, respectively, compared to hydrolysis in the presence of trimethylamine. In addition, the isotope effect, which was expected for

general acid catalysis, was observed when H_2O was replaced by D_2O ($k_H : k_D = 1.6$ for the diamine **23a**). The diamines **23a–c** in the neutral form exhibit substantially higher catalytic activity (1400–2040 times higher than that of trimethylamine), the isotope effect being absent. Apparently, in the latter case, the positive charge that arises in the course of the nucleophilic attack at the phosphorus atom (the transition state **B**) is electrostatically stabilised by the free electron pair of the second nitrogen atom.[145]

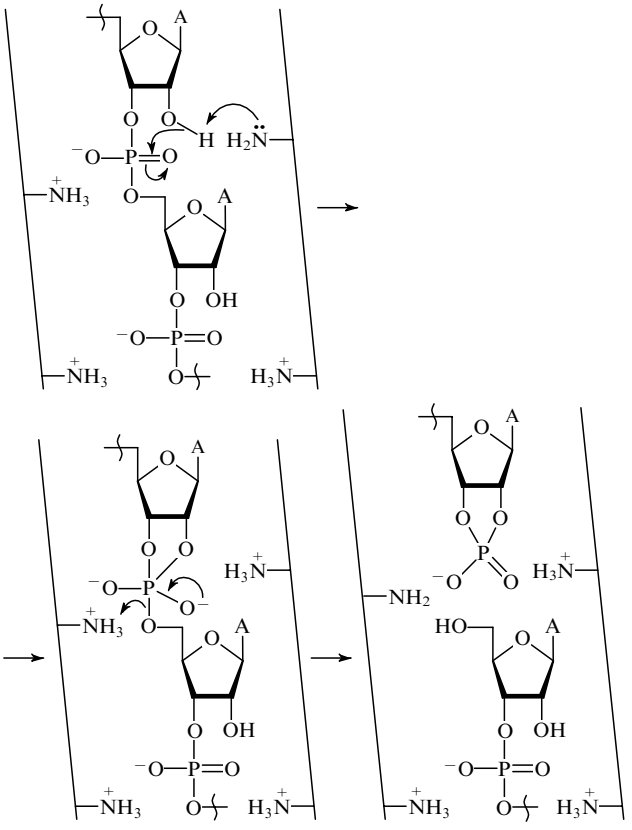


A **B**

R = 4-$NO_2C_6H_4$, 2,4-$(NO_2)_2C_6H_3$.

Hydrolysis of poly(A), poly(C) and poly(U) in solutions of polyamines (1–2 mol litre^{-1}), such as ethylenediamine, triethylenetetramine, *etc*., was investigated.[146] It appeared that ethylenediamine possesses the highest catalytic activity, the efficiency of hydrolysis (50 °C, 2 days) in a solution of ethylenediamine (1 mol litre^{-1}) being decreased in the series poly(A) > poly(U) > poly(C) (33%, 28% and 16%, respectively). Under these conditions, poly(G) was not hydrolysed.[147]

Apparently, the weak hydrolytic activity of bleomycin (metal-dependent antibiotic) in the absence of metal ions is explained by the presence of aliphatic amino groups.[148] Regular peptides containing arginine and(or) lysine can accelerate hydrolysis of the phosphodiester bonds in ApAp and poly(A).[149–151] The regular peptides poly(Leu-Lys-Lys-Leu) and poly(Leu-Lys) capable of forming α-helices and β-folds, respectively, exhibit the highest activity in hydrolysis. In their complexes with RNA, the ratio $Lys^+ : PO_4^- = 2 : 1$ was achieved. Distortion of the polypeptide structure by introducing proline (Pro-Leu-Lys-Leu-Lys) or with the use of the D,L-isomers (D,L-Leu-D,L-Lys) leads to a sharp decrease in the catalytic activity of peptides. It should be noted that hydrolysis is not accelerated in the presence of poly(His), poly(Arg-Glu-Glu) and a number of other regular peptides containing various functional groups. No activity was also observed for the Ac-Leu-Lys-NHEt dipeptides. Apparently, the fact that the distances between the phosphate residues in the ribonucleotide (6.2 Å) coincide with the distances between the positive charges in poly(Leu-Lys-Lys-Leu) (6 Å) and poly(Leu-Lys) (6.9 Å) plays a decisive role in the manifestation of the catalytic activity exhibited by the above-mentioned peptides in RNA hydrolysis (Fig. 3).[152, 153]



A, heterocyclic base residue.

Figure 3. The assumed mechanism of hydrolytic RNA cleavage under the action of polycharged peptides.

On the whole, both polyamines and regular peptides ehxibit low hydrolytic activity. Thus, the degree of depolymerisation of ApAp under the action of the most active regular peptide, *viz*., poly(Leu-Lys), at 50 °C (pH 8.0) was 85% after 7 days.[150] After 2 days, the degree of depolymerisation of poly(A), poly(U) and poly(C) under the action of the most active polyamines at 50 °C (pH 8.0) varied from 16% to 69%.[147]

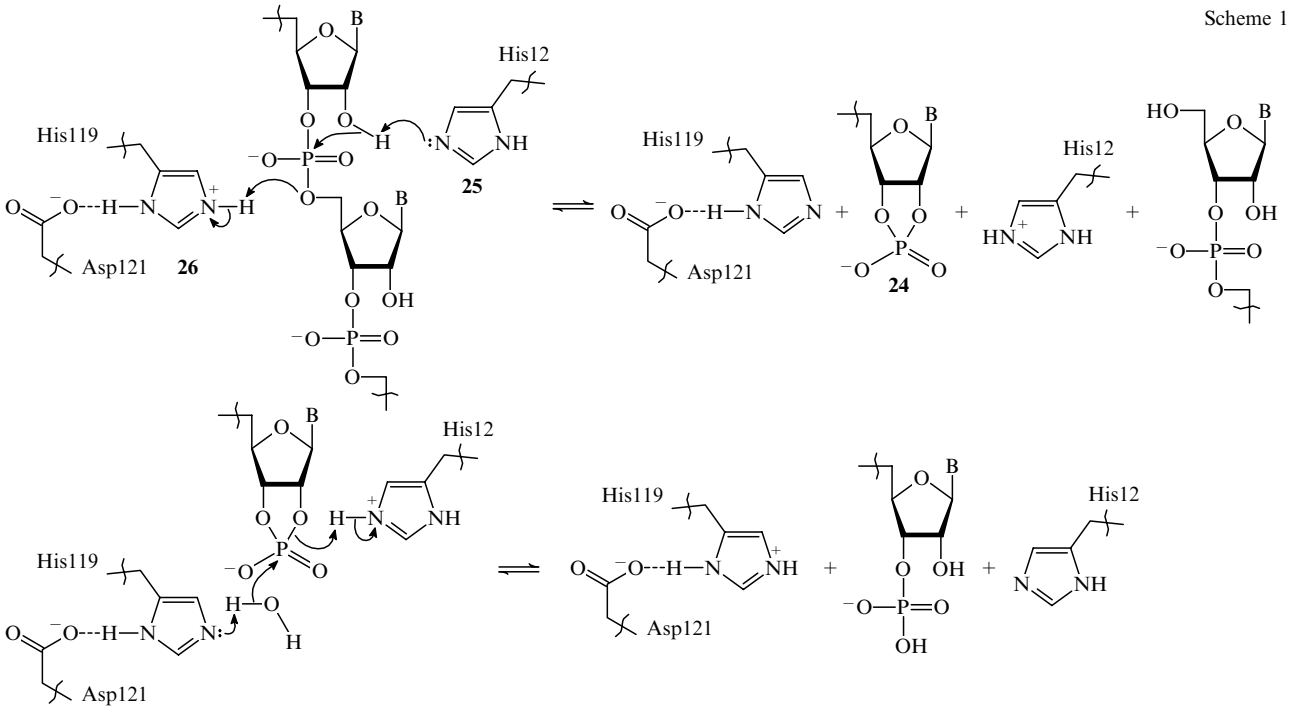
5. Reagents modelling active centres of metal-independent ribonucleases

Of metal-independent ribonucleases, ribonucleases A and T1 have received the most study. Both enzymes were found to cleave phosphodiester bonds in RNA according to the mechanism of intramolecular transesterification under conditions of acid–base catalysis. The scheme of the 'classical' two-step mechanism of acid–base catalytic hydrolysis of phosphodiester bonds in ribonucleic acids under the action of RNase A involves transesterification to form cyclic phosphodiester **24** followed by its hydrolysis. The abstraction of the proton from the 2′-hydroxyl group of the ribose residue under the action of the base **25** and the transfer of the proton from the fragment **26** to the oxygen atom of the leaving group are the key stages of this mechanism (Scheme 1).[154, 155] The details of interactions of the amino acid residues in the active centre both with each other and with the substrate have consistently been refined.[156–159]

Breslow and coworkers[160] proved that the active centres of ribonulceases can, in principle, be modelled by small molecules. The authors found that RNA hydrolysis was catalysed by a neutral concentrated (1–2 mol litre^{-1}) imidazole buffer and then synthesised a conjugate of cyclodextrin with molecules containing imidazole fragments. This conjugate catalysed hydrolysis of a model substrate, *viz*., *tert*-butylcatechol 1,2-cyclophosphate.[161]

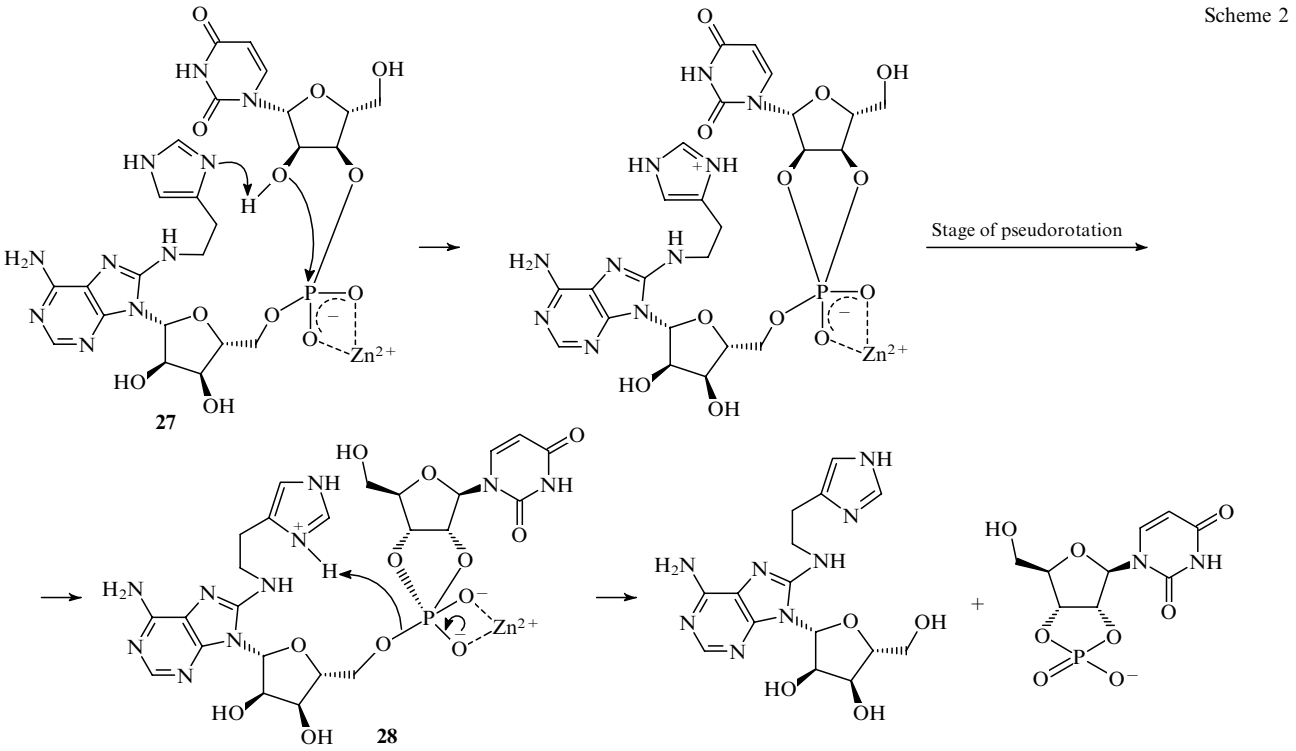
Hydrolysis of RNA with imidazole conjugates or an imidazole buffer proceeded most efficiently at pH 7, which corresponds, as in the case of ribonuclease A, to the mechanism of general acid–base catalysis, *i.e*., one protonated and one nonprotonated imidazole fragment were involved in hydrolysis. Breslow examined three possible bisimidazole conjugates of cyclodextrin and found that only one of them, which is characterised by the spatial arrangement of the imidazole rings most favourable for the cleavage of the phosphodiester bond, exhibited catalytic activity in hydrolysis of the model phosphodiester.[162] Some conjugates of cyclodextrin containing various combinations of the imidazole and amino groups, cyclic and acyclic ethers and metal complexes also possess phosphoesterase activity with respect to model substrates.[163–166] Prakash *et al*.[167] proposed a structure modelling ribonuclease A in which the same imidazole fragment acts as a base and a proton donor. The model proved to be suitable in the presence of zinc ions, which stabilise the transition state and cause an increase in the electrophilicity of the phosphorus atom (structure **27**, Scheme 2). The authors believed[167] that hydrolytic cleavage of the phosphodiester bond in the diribonucleotide proceeds according to the related transfer mechanism (Section III.1). It was assumed that the leaving group in the structure **27** is located rather close to the 2′-hydroxyl group. The proton transfer from the 2′-hydroxyl group of the substrate to the

Scheme 1



B, heterocyclic base residue.

Scheme 2



imidazole fragment and the stage of pseudorotation are followed by the cleavage of the phosphodiester bond involving a proton of the same imidazole fragment (intermediate **28**). This mechanism requires an additional stage of pseudorotation; however, the process can involve the same histamine fragment as proton donor and proton acceptor.

More recently, hydrolytic RNA cleavage under the action of imidazole and its conjugates with cationic and intercalating compounds has been studied in detail.[168, 169] It was demonstrated that these compounds can be used as structural probes in studies of the secondary structures of RNA and RNA–deoxyoligonucleotide complexes in solutions.[168, 169]

Hydrolysis with both an imidazole buffer and imidazole conjugates proceeds predominantly in single-stranded regions of RNA. Apparently, this is associated with the lower conformational flexibility of nucleotides in double-stranded regions and the less favourable spatial arrangement of the 2′-hydroxyl group with respect to the phosphorus atom of the internucleotide phosphate group. It should be noted that the rate of hydrolysis with an imidazole buffer is independent of the nucleotide sequence in the region subjected to cleavage (Fig. 4), whereas imidazole conjugates have a pronounced tendency for cleavage at the pyrimidine – purine regions.[168]

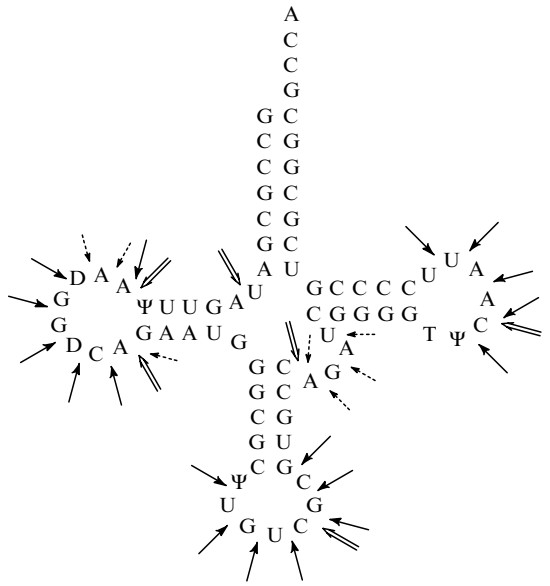
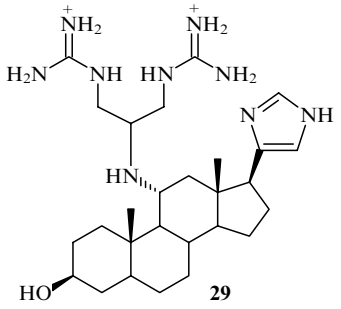


Figure 4. Structure and major cleavage sites of $tRNA^{Asp}$.
The most probable and less probable sites of cleavage under the action of an imidazole buffer are indicated by single and dashed arrows; the sites of cleavage under the action of imidazole conjugates are indicated by doubled arrows.

Modelling of the active centre of staphylococcus nuclease (nuclease S) is also of interest. The structure and the mechanism of action of nuclease S were established based on X-ray diffraction data, results of kinetic studies and data on substitutions of amino acids in the active centre of the enzyme.[170–172]

It is assumed that due to coordination by the guanidinium groups, the phosphate ion adopts the conformation favourable for hydrolysis with nuclease S. Simultaneously, the five-coordinate intermediate state of the phosphorus atom is stabilised. Although being a metal-dependent enzyme, nuclease S, like other compounds containing guanidinium groups, can be bound to the phosphate anions[173,174] and accelerate hydrolysis of the phosphodiester bonds in the absence of metal ions.[175] Hydrolysis of phosphodiester bonds under the action of compounds containing the guanidinium fragments is accelerated in the presence of external or internal nucleophiles.[176,177] Yet another type of hydrolytic centre combining elements of the active centre of RNase A (general base catalysis with imidazole) and nuclease S (coordination of the phosphate anion and stabilisation of the five-coordinate state of the phosphorus atom) was proposed by Oost and Kalesse.[178] The compounds synthesised contained the imidazole fragment and one or two guanidinium groups bound to a steroid core through spacers of different lengths. Most of the compounds synthesised exhibited low hydrolytic activity with respect to the model substrate, *viz*., 2′-hydroxypropyl 4-nitrophenyl phosphate. The efficiency of hydrolysis depended on both the number of guanidinium groups (two groups were better than one group) and their positions with respect to the steroid rings as well as on the length of the linker. The most efficient construction, *viz*., **29**, also exhibited a low hydrolytic activity with respect to UpU ($k = 2.4 \times 10^{-4}$ h^{-1}, 60 °C, pH 7.5). At the same time, simpler compounds containing only guanidinium groups [1,3-bis(guanidinium)propane] or simultaneously guanidinium and imidazole fragments {1,3-bis(guanidinium)-2[2-(1*H*-imidazol-4-yl)ethylamino]propane} appeared to be hydrolytically inactive.



29

Therefore, functional models of the active centres of natural ribonucleases can be, apparently, obtained by constructing molecules which contain basic and acidic catalytic centres adopting particular orientations.

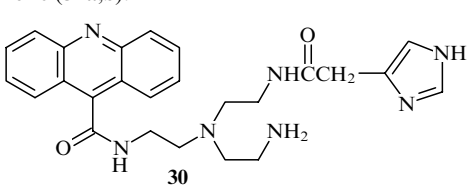
Most often, synthetic ribonucleases contain imidazole residues (an analogue of His12 in RNase A) or the carboxylate anion (an analogue of Glu58 in RNase T1) as a base and the protonated imidazole fragment as a proton donor (analogously to His119 and His92, respectively). Apparently, efficiency of catalysis can be improved by introducing positively charged groups (amino or guanidinium) into the catalytic centre to achieve a particular orientation of the phosphate ion with respect to the catalytic centres and to stabilise the five-coordinate intermediate state of the phosphorus atom. Probably, the introduction of the nucleophilic group (for example, the hydroxyl group simulating the 2′-hydroxyl group of ribose) into the reactive centre will allow one to construct catalysts hydrolysing phosphodiester bonds in DNA.[179]

IV. Site-specific RNA-cleaving reagents

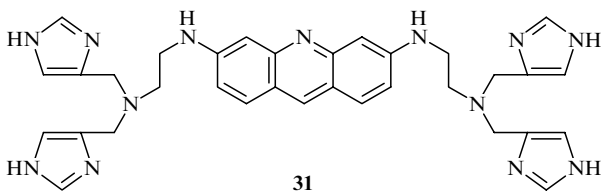
Site-specific RNA cleavage can be performed with the use of reagents containing groups which form complexes with particular nucleotide sequences or elements of the three-dimensional RNA structure. The most evident method for solving this problem is the use of oligonucleotides complementary to the predetermined primary sequences of the RNA target. In addition, a number of low-molecular-weight ligands possessing affinity for particular spatial structural elements of RNA are available.

1. Conjugates based on polycyclic compounds

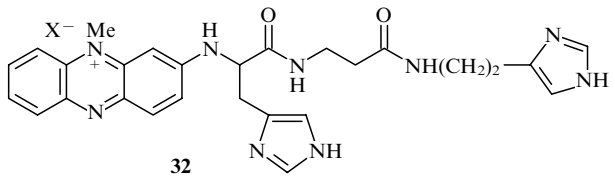
Catalytically active groups can be added to polyaromatic compounds capable of intercalating into double-stranded regions of nucleic acids *via* flexible linkers. Synthetic constructions containing polycyclic intercalating compounds and metal complexes are widely used for DNA cleavage.[180,181] Efforts were made to prepare compounds which can hydrolyse RNA, by adding various combinations of guanidinium, amino and carboxyl groups to intercalating compounds. Some most active representatives of such conjugates were synthesised based on acridine (**30** and **31**),[182–184] phenazine (**32**),[185] isoalloxazine (**33**)[186] and anthraquinone (**34a**,**b**).[187]



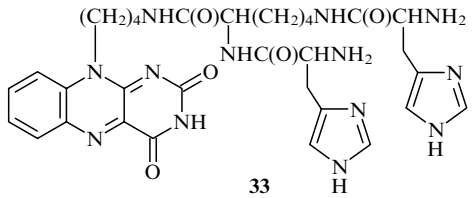
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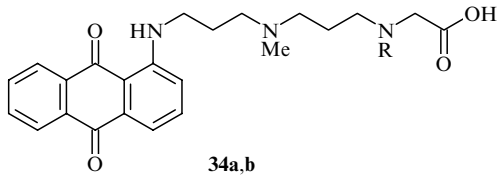
31



32



33



34a,**b**

R = H (**a**), CH_2CO_2H (**b**).

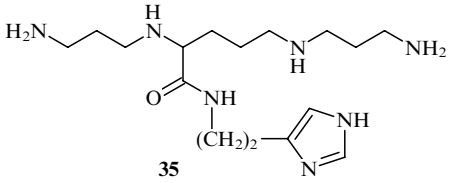
The substrates (tRNA, mRNA and synthetic oligonucleotides) employed did not enable one to perform a reliable comparison of the catalytic activity of the conjugates **30**–**34**. Generally, the catalytic activity is determined based on the disappearance of the initial nucleotide-containing substance without analysis of the hydrolysis products. This procedure does not allow one to reveal relationships between the structures of synthetic ribonucleases and their catalytic activity. We synthesised a series of compounds, which differ by both the structure of the linkers and the combination of reactive groups in the catalytic centre.[186] The phenazine conjugates whose RNA-hydrolysing centres contained two imidazole fragments, which were separated from the intercalator by the linker consisting of 8–14 C—C or C—N bonds, possessed the highest activity. The compounds containing the imidazolyl fragment and the carboxyl group exhibited somewhat lower activity.

The conjugates **30**–**32** and **34a** exhibited pronounced specificity with respect to cleavage of the CpA and UpA sequences in single-stranded RNA regions.[185,186] The exceptions are the isoalloxazine conjugate **33** (see Ref. 186) and the anthraquinone conjugate **34b** (see Ref. 187) containing two carboxyl groups. Hydrolysis of $tRNA^{Phe}$ in the presence of the anthraquinone derivative **34a** containing one carboxyl group proceeded predominantly at the CpA regions. However, when one more carboxyl group was introduced into the RNA-hydrolysing centre (the compound **34b**), the phosphodiester bonds in the GpA and GpG sequences were predominantly subjected to hydrolysis, and GpC was hydrolyzed to a lesser degree.

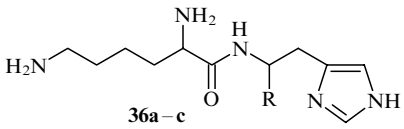
The low activity of most of the conjugates based on polycyclic compounds is attributable to the fact that these conjugates are inserted between RNA turns thus stabilising its double-stranded structure. In this case, the catalytic groups are located in the RNA region most resistant to hydrolysis. The use of long linkers makes possible localisation of the catalytic groups in the region of more labile single-stranded RNA fragments, which, in turn, provides higher efficiency of hydrolysis.[187]

2. Conjugates based on polycations

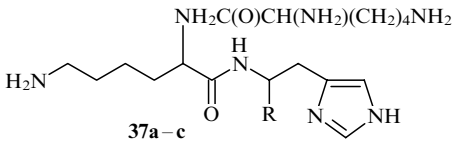
Unlike conjugates based on polycyclic compounds, which tend to be bound to double-stranded fragments of nucleic acids, conjugates based on cationic compounds are bound to RNA through Coulomb interactions, thus providing their homogeneous distribution over all sterically accessible phosphodiester bonds of the RNA target. Conjugates **35**–**39** based on synthetic and natural polyamines containing the imidazole and 1,4-diaminobutane fragments as the RNA-hydrolysing groups were described.[188–192]



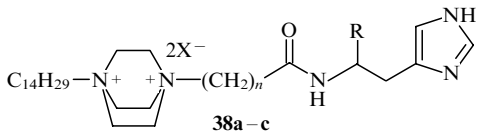
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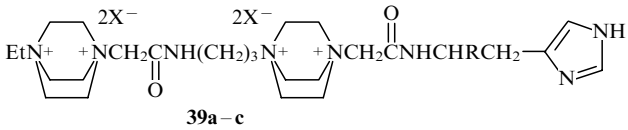
36a–**c**



37a–**c**



38a–**c**



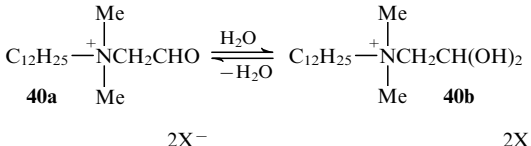
39a–**c**

35–**39**: R = H (**a**), CO_2H (**b**), CO_2Me (**c**).

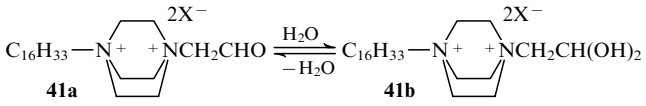
As mentioned above, 1,4-diaminobutane and imidazole at concentrations of 1–2 mol litre^{-1} promote RNA hydrolysis. Synthetic ribonucleases **35**–**39** based on conjugates of imidazole with polyamines efficiently catalyse hydrolysis at substantially lower concentrations (10^{-3}–10^{-5} mol litre^{-1}), which is indicative of the synergistic action of the individual elements of these constructions. A sharp decreases in the efficiency of catalysis on going from imidazole conjugates to *N*-methylimidazole conjugates [188] and the manifestation of an isotope effect (k_H/k_D = 2.28) upon hydrolysis of dinucleosidemonophosphate CpA in water and D_2O (see Ref. 169) indicate that acid–base catalysis occurs at the limiting reaction stage. The catalytic activity of the compounds **35**–**39** was investigated in experiments with synthetic oligonucleotides, various tRNA and fragments of virus RNA. The conjugates **35**–**39** were demonstrated to provide high rates of cleavage of the phosphodiester bonds in the 5′-Py-A motifs in single-stranded RNA regions.[188–190] It should be noted that no preferential binding of the conjugates to phosphates of the above-mentioned motifs compared to other internucleotide phosphates was assumed. The conjugates studied contained different RNA-binding fragments, while the RNA targets had different structures. Hence, high instability of pyrimidine–purine sequences in RNA can be explained only by their characteristic features. The conjugates **35**–**39**, which are able to perform efficient and specific depolymerisation of RNA under mild conditions, can be considered as an alternative for RNase A in studies of the secondary structure of RNA.[168,188]

Menger and coworkers [193,194] proposed to carry out hydrolytic cleavage of phosphodiester bonds in RNA with the use of compounds **40b** and **41b**, which, unlike the conjugates **35**–**39**, are nucleophilic catalysts. Due to the electronegative substituent in the β position, the aldehyde groups in the compounds **40a** and **41a** are hydrogenated in weakly alkaline and neutral solutions to give the highly nucleophilic catalysts **40b** and **41b**. Owing to the

positive charge of the nitrogen-containing fragment, the nucleophilic group is localised in the vicinity of the phosphodiester bond. The authors believed that the presence of the alkyl substituent leads to the enhancement of the efficiency of hydrolysis due to micellar catalysis (by analogy with the micellar analogues of serine proteases proposed previously [195]). The catalysts **40** and **41** accelerated hydrolysis of the model compound, *viz*., of bis-(4-nitrophenyl) phosphate, by a factor of 1800 and 750, respectively, compared to the rate of hydrolysis in the absence of catalysts. It should be noted that micellar catalysis led to acceleration by only a factor of 210.[193, 194] Since examples of cleavage of natural RNA substrates in the presence of catalysts of this type are lacking in the literature, the efficiency of these catalysts cannot be compared with that of compounds functioning based on the general acid – base catalysis.

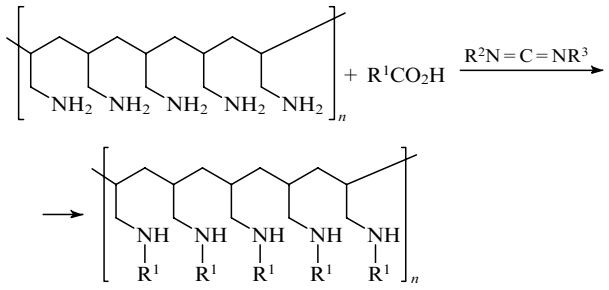


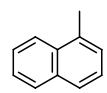
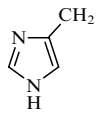
40a **40b**



41a **41b**

Catalysts for cleavage of phosphodiester bonds based on polyamines were constructed using methods of combinatorial chemistry.[196] A mixture of several carboxylic acids was activated by water-soluble carbodiimide in the presence of polyallylamine with a molecular weight of 30 000 – 40 000. The ratio between the components was chosen so that the total degree of acylation of the amino groups of the polymer was 5% – 45%. A large library of water-soluble polymers with a statistical arrangement of functional groups was obtained by varying both the ratio between the carboxylic acids in the reaction mixture and the degree of modification of the initial polyamine.



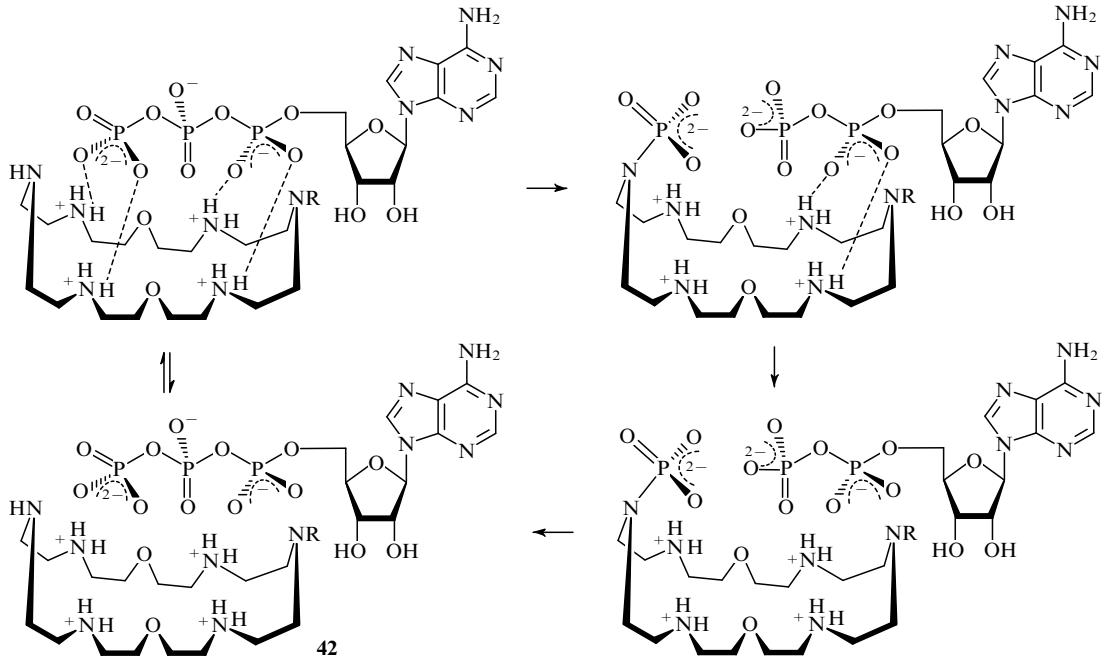
R^1 = HO_2C, 4-HOC_6H_4, , , $(CH_2)_2NHC(=NH)NH_2$,

$HOCH_2$, $HS(CH_2)_2$, C_7H_{15}; R^2 = Et, R^3 = $(CH_2)_3NMe_2$.

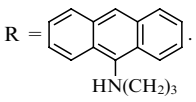
The catalytic activity of the compounds synthesised was determined from the degree of hydrolysis of bis(4-nitrophenyl) phosphate in the presence of metal ions (Mg^{2+}, Zn^{2+} or Fe^{3+}). This approach made it possible to reveal a series of polymers accelerating hydrolysis by a factor of 20 000 – 30 000. Polyamine containing 7.5% of caprylic-acid residues, 15% of imidazolylacetic-acid residues and 10% of 4-hydroxybenzoic-acid residues in the presence of iron ions proved to be most active (k_{cat}/k = 31 400).[197] The drawback of this approach is the lack of information on the structures of catalytically active sites. At the same time, methods of combinatorial chemistry make possible the rapid determination of functional groups necessary for particular catalytic processes to proceed efficiently.

Synthetic constructions based on macrocyclic polyamines are of particular interest. In these compounds, the catalytic and affine centres merge together. The efficiency of binding of macrocycles with RNA can be additionally enhanced by addition of the former to polyaromatic compounds.[198] The cleavage of the phosphodiester bond in adenosine-5′-triphosphate catalysed by macrocyclic polyamine **42** is presented in Scheme 3. Compounds of the type **42** did not exhibit hydrolytic activity with respect to nucleic

Scheme 3



42

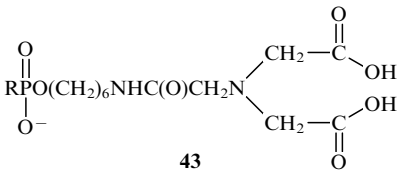
R = .

acids.[199] However, the construction of synthetic catalysts based on molecules whose functional groups have a limited degree of freedom shows considerable promise.

3. Conjugates based on oligonucleotides

In the construction of oligonucleotide conjugates, virtually all types of the above-described catalytic groups were used (see reviews [200–204]). These groups were introduced into the midpoint of the oligonucleotide address (Fig. 5 *a*) or were added to the terminal fragments of the nucleotides (Fig. 5 *b*). The first mode of construction of conjugates seems to be attractive because subsequent RNA cleavage under the action of these conjugates leads to a substantial decrease in stability of the complexes of the conjugate with the substrate, which should be favourable for hydrolysis under the catalytic conditions. At the same time, the catalytic group in the complex of RNA with such a conjugate is located in the region of the duplex structure characterised by high stability.[102] When located at the terminus of the oligonucleotide, the catalytic group in the resulting complex is oriented toward the more labile single-stranded structure. However, the stability of the complex after RNA cleavage remains virtually unchanged. Therefore, exchange of the conjugate between depolymerised RNA and intact RNA is hindered, which should retard efficient functioning of the catalysts.

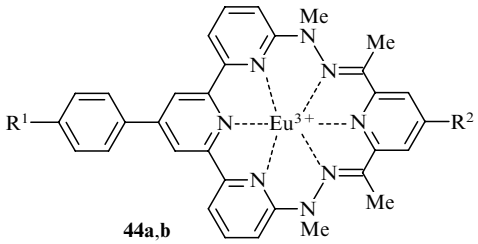
Conjugates containing catalytic groups attached to the 5′-terminal phosphate group differ substantially in efficiency of RNA depolymerisation. For the Lu^{3+} complex, which was prepared *in situ* based on oligo-DNA containing the iminodiacetic-acid residue (**43**) at the 5′-terminus, the degree of polymerisation was 17%



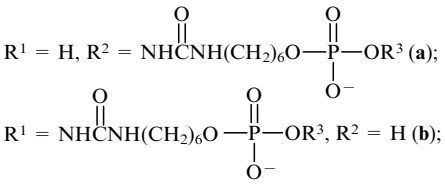
43

R, oligonucleotide residue.

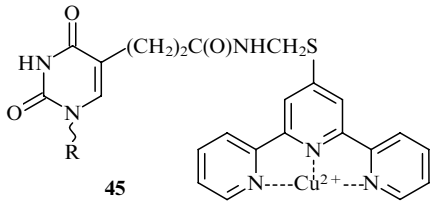
(37 °C, 8 h). In the case of conjugates based on macrocyclic Eu^{3+} complexes **44a**,**b**, the degree of depolymerisation was 30% (37 °C, 18 h) and 80% – 90% (37 °C, 16 h), respectively.[206] The introduction of the same metal complexes at position 5 of thymidine located at the midpoint of the oligonucleotide address gave rise to inactive [205] or weakly active [206] conjugates. The catalytic activity of oligonucleotide conjugates **45** containing copper complexes at position 5 of thymidine also appeared to be low. Thus the degree of cleavage upon incubation of RNA with such conjugates at 45 °C for 72 h was at most 18% – 25%.[207]



44a,**b**



R^3, oligonucleotide residue.



45

R, oligonucleotide residue.

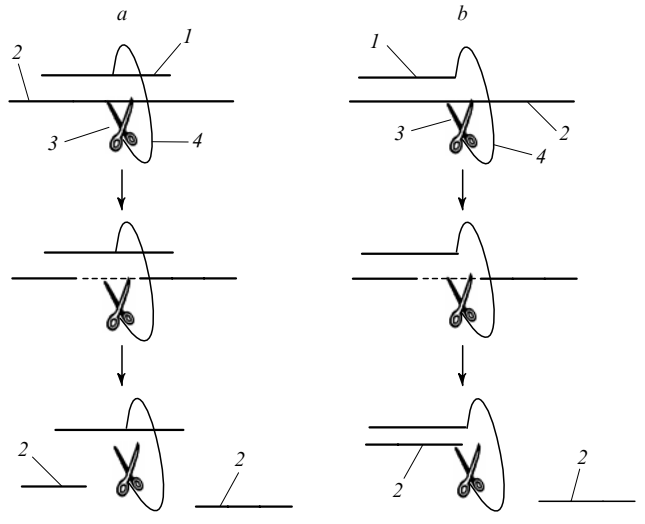


Figure 5. Main approaches to the construction of superspecific synthetic ribonucleases based on oligonucleotide conjugates;
(*a*) introduction of catalytic groups at the midpoint of the oligonucleotide address, (*b*) the addition to the terminal fragment; (*1*) is an oligonucleotide, (*2*) is an RNA target, (*3*) is a catalytic group, (*4*) is a linker.

High efficiency of cleavage is achieved when hydrolytic groups are located in the region of loop structures of the RNA target (Fig. 6 *a*). As mentioned above, phosphodiester bonds in loop regions of nucleic acids are characterised by high sensitivity with respect to cleavage under the action of reagents of different nature.[72, 103, 104, 208, 209] The efficiency of RNA cleavage with conjugates forming loop structures with RNA was as high as 92%, whereas that in analogous reactions involving conjugates forming perfect complexes was at most 7%.[205]

In the case of addition of a metal complex at the 5′-terminal phosphate group of the oligonucleotide address, hydrolysis of phosphodiester bonds occurs at a distance from one to seven nucleotide units depending on the type of the linker.[204, 210] In the case of formation of an artificial loop in the vicinity of the 5′-terminus, the cleavage is virtually quantitatively reoriented from the single-stranded region to the loop, the total degree of hydrolysis being increased.[205] It was demonstrated that synthetic ribonucleases based on 12-unit 2′-modified oligoribonucleotides, which were constructed on the above-described principle, can execute up to 40 events of RNA cleavage in the course of incubation at 37 °C during 64 h (at a reagent concentration of 1×10^{-3} mol litre^{-1}) in the presence of a 50-fold excess of RNA.[211]

Yet another way of increasing the flexibility of the sugar-phosphate core in the target RNA region is based on the replacement of one nucleotide unit in the oligonucleotide address by a non-nucleotide insertion.[212]

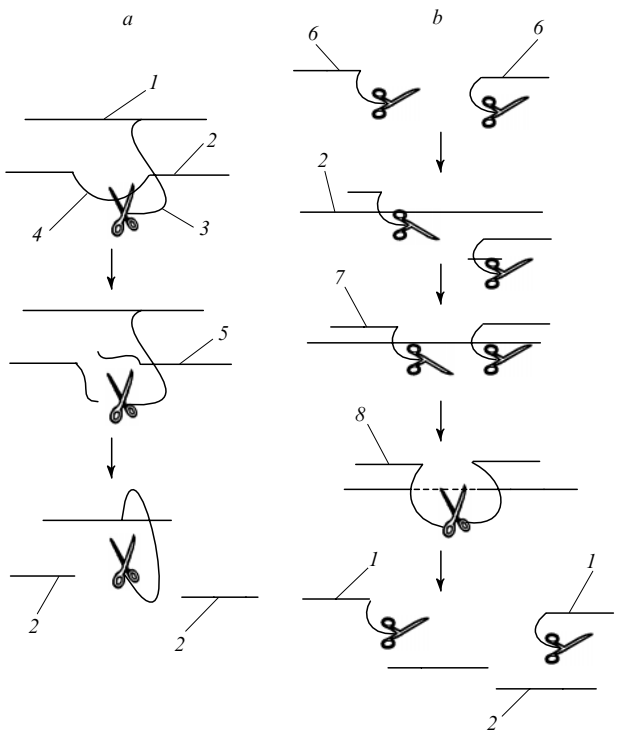
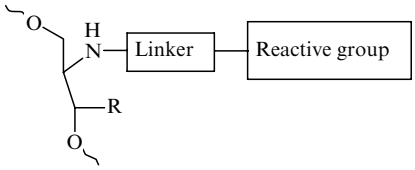
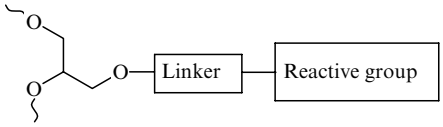


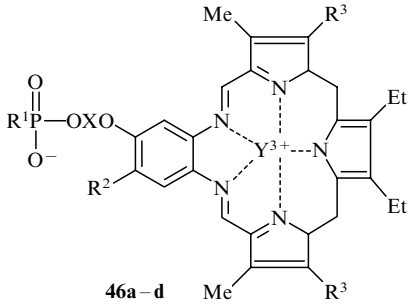
Figure 6. Principles of construction of synthetic ribonucleases based on conjugates containing noncomplementary regions (*a*) and based on module conjugates (*b*);
(*1*) is oligonucleotide, (*2*) is an RNA target, (*3*) is a linker, (*4*) is a noncomplementary region, (*5*) RNA cleavage, (*6*) are oligonucleotides containing components of the catalytic centre, (*7*), formation of the catalytic centre at the RNA target, (*8*) RNA cleavage.



R = H, Me.



The ability of these oligonucleotide conjugates to cleave RNA under catalytic conditions was exemplified by conjugates **46a** containing macrocyclic Dy^{3+} complexes.[213]



R^1 is the oligonucleotide residue; R^2 = H, OMe; R^3 = $(CH_2)_3OH$, Et; X = $(CH_2)_3$, $(CH_2)_6$, $(CH_2)_6NHCOCH_2$; Y = Dy (**a**), La (**b**), Lu (**c**), Eu (**d**).

A promising approach to the construction of RNA-cleaving oligonucleotide conjugates involves the design of module (binary) reagents (see Fig. 6 *b*). The approach is based on separation of the catalytic centre into two fragments and the synthesis of conjugates of these fragments with oligonucleotides complementary to the adjacent regions of the RNA target. The catalytic centre arises only in the case of formation of a proper ternary complex in which two inactive structures are recombined to form a catalytically active centre. This centre can be formed either by individual reactive groups involved in natural ribonucleases (for example, two imidazole fragments) or by poorly reactive metal complexes forming more reactive binuclear catalytic centres. Undoubtedly, this approach imposes much more rigid limitations on both the structural parameters of the fragments of the reactive centre and the geometry of the conjugates as a whole. However, this approach has a number of important advantages, *viz*., it makes possible the improvement in specificity of the reaction with the target sequence and allows one to exclude side reactions of the conjugates with non-target biopolymers.

The most efficient conjugates of **46a–d** contain complexes of rare-earth metals (La^{3+}, Lu^{3+}, Eu^{3+} or Dy^{3+}) as catalytic groups. Dissociation of these ions from the metal complexes can lead to nonspecific cleavage of other biopolymers resulting in their high toxicity in biological systems. Recently, binuclear zinc complexes have been proposed for solution of this problem. Under physiological conditions, zinc ions by themselves cannot efficiently catalyse depolymerisation of biopolymers, whereas the efficiency of their binuclear complexes is rather high.[214]

Reagents based on oligonucleotide conjugates with catalytic groups, which do not contain metal ions, seem to be more promising because they allow one to exclude completely any cofactors. In combination with high specificity of the oligonucleotide address, the above fact may enable one to prepare compounds capable of performing efficient cleavage of the target RNA sequences leaving other biopolymers intact. However, some groups (amino and carboxyl groups, heterocyclic fragments) exhibit substantially lower catalytic activity than metal ions and metal complexes. High efficiency of hydrolysis in the presence of organic compounds is achieved only in the case of the optimum spatial arrangement of all groups involved in the catalytic process. Oligonucleotide derivatives containing ethylene-[215] or propylenediamine[216] at the 5′-terminus are the simplest RNA-cleaving conjugates, which do not contain metal ions. Cleavage of $tRNA^{Phe}$ under the action of a 19-unit oligonucleotide conjugate containing the diamine residue was reported.[217] The efficiency of this reaction was 10% after incubation at 50 °C for 4 h. Similar efficiency was observed upon cleavage of a 30-unit model oligoribonulceotide under the action of an analogous conjugate.[218] In both cases, the 5′-CpA sequences in the region of the reagent localisation were subjected to hydrolysis. The introduction of oligoamines through a non-nucleotide insertion at the midpoint of the conjugate leads to a decrease in efficiency of hydrolysis under analogous conditions to 3%.[219] At the same time, the degree of hydrolysis performed at 40 °C for 4 h with the use of a 10-unit oligopeptidylnucleotide containing diethylenetriamine at the 5′-terminus instead of the 19-unit oligodeoxyribonucleotide was 30%, and hydrolysis was quantitative after 24 h.[220] The target used in the latter study differed from that employed in the study [217] by the single substitution G → C as a result of which the CpApCpA fragment was present in the region of location of the hydrolytic group. Both bonds in CpA were subjected to hydrolysis, but to a different degree.

In the absence of metal ions, conjugates containing fragments, which bear the 1,4-diaminobutyl, *N*-propylimidazolium or histamine residues at the 3′-terminal phosphates of oligonucleotides, do not exhibit catalytic activity in hydrolysis of the model oligoribonucleotide. The histamine-containing compound exhibited weak activity upon introduction of zinc ions into the reaction mixture.[221] In a latter study, the polyuridyl sequence was used as the target.

The synthesis of oligonucleotide conjugates containing the $(Leu\text{-}Arg)_nGlyNH_2$ oligopeptides (n = 2–4) at the 5′-terminal

phosphate was reported.[222, 223] Incubation of a model 14-unit oligoribonucleotide with the complementary six-unit oligodeoxyribonucleotide whose oligopeptide fragment consists of alternating Leu-Arg pairs led to cleavage of the RNA target. In the case of the conjugate containing three Leu-Arg pairs, prolonged incubation (48 h, 37 °C) resulted in insignificant cleavage (~5%) of the target at the CpU site in the vicinity of the localisation site of the oligopeptide. In experiments with conjugates bearing two or four Leu-Arg pairs, the efficiency of cleavage under these conditions reached 30% and 70%, respectively. In the latter case, two GpA sequences and, to a lesser extent, UpU located in the double-stranded region of the complex were the major cleavage sites. In the case of incubation of *E. coli* $tRNA^{Phe}$ with conjugates based on the pTCAATC oligonucleotide, which is complementary to two sequences in the anticodon and TΨC loops, the phosphodiester bonds in the region of the D loop, in the TΨC loop and, to a lesser extent, in the anticodon region of this tRNA were cleaved. Cleavage of the phosphodiester bonds in the non-target region of the D loop was related to the possible spatial proximity to the TΨC loop possessing the partial binding site of the reagent.[222, 223] Unlike the model 14-unit oligoribonucleotide, the site of tRNA subjected to hydrolysis under the action of the reagent ($n = 3$ or 4) was independent of the length of the peptide residue, although the degree of hydrolysis of the target was also substantially decreased on going from $n = 4$ to $n = 3$. The maximum efficiency of hydrolysis was 80% ($n = 4$, 48 h, 20 °C).

We prepared oligonucleotide conjugates containing the bisimidazolium fragments at the 5′- or 3′-terminal phosphate.[224] The activity of the conjugates was tested using yeast phenylalanine tRNA as well as pre- and mini-exons of mRNA from *Leishmania amazonensis*. In the case of $tRNA^{Phe}$, the C(61)A(62) and C(63)A(64) sequences located in the vicinity of the hydrolytically active groups were subjected to cleavage, in all cases the highest efficiency of cleavage being observed at the CpA bond separated from the binding site of the reagent by three nucleotide units. The reagent containing the catalytic group at the 3′-terminal phosphate appeared to be somewhat more active than its 5′-analogue. After incubation at 37 °C for 8 h, the total efficiency of tRNA cleavage at three sites reached 60%.[225] In the case of the mini-exon of mRNA from *Leishmania amazonensis*, the efficiency of hydrolysis for the 17-unit oligonucleotide containing the bisimidazolium fragment at the 3′-terminus was 100% after 5 h. Cleavage proceeded primarily at the CpA sequences (80%) in the region of the expected localisation of the reactive groups. Similar results were obtained for oligonucleotide conjugates whose 5′-terminal phosphates bear synthetic fragments containing the imidazole fragment and the aliphatic amino group.[226] An increase in the length of the spacer, which links the bisimidazole fragments with the oligonucleotide address, as well as the introduction of reactive groups through a non-complementary nucleotide residue led to substantial enhancement of the efficiency of hydrolysis. Incubation of $tRNA^{Phe}$ with such conjugates at 37 °C for 3–5 h resulted in quantitative RNA cleavage (Fig. 7). Hydrolysis was completely inhibited in the presence of an oligonucleotide complementary to the cleavage sites (indicated by arrows, the oligonucleotide A, see Fig. 7).[227] Dendrimeric oligonucleotide conjugates containing from 2 to 24 imidazole residues were proposed.[228] This approach seems to be attractive for the synthesis of libraries of oligonucleotide conjugates containing not only imidazole fragments, but also other functional groups typical of the active centres of natural enzymes.

Methods of combinatorial chemistry can be used not only for the construction of reactive groups, but also for the choice of the RNA region most favourable for hydrolysis. The synthesis of a library of 8-unit 2′-*O*-modified oligoribonucleotides with random sequences was reported.[229] Each oligonucleotide contains the Eu^{3+} complex **44** at the 5′-terminus. Four hydrolysis sites were found after incubation of this library with 39-unit RNA (1 mmol litre^{-1} of the conjugates, 50 nmol litre^{-1} of RNA, 37 °C) for even 2 h. This result was unexpected because the concentration of each particular conjugate in the reaction mixture was no higher than 15 pmol litre^{-1}.

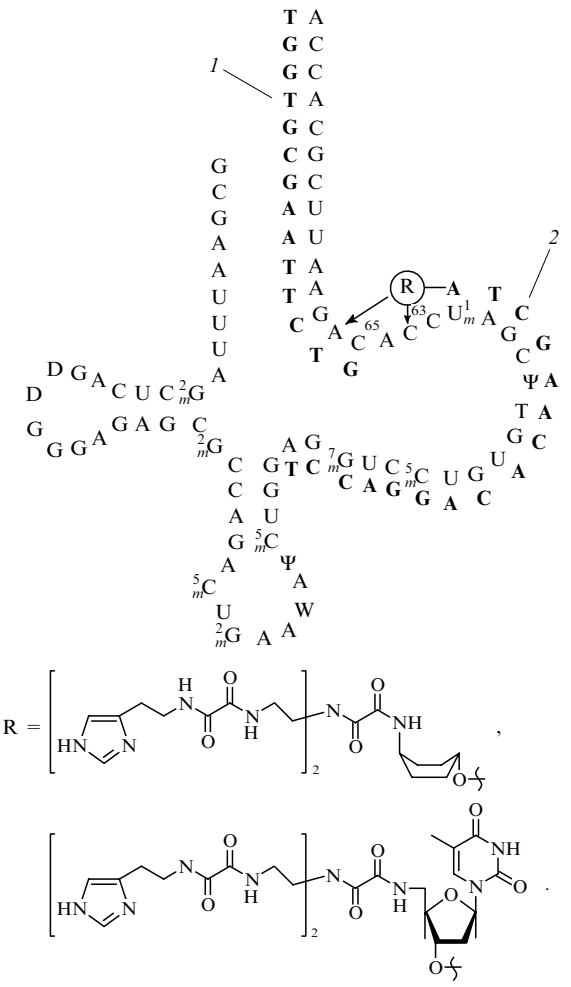
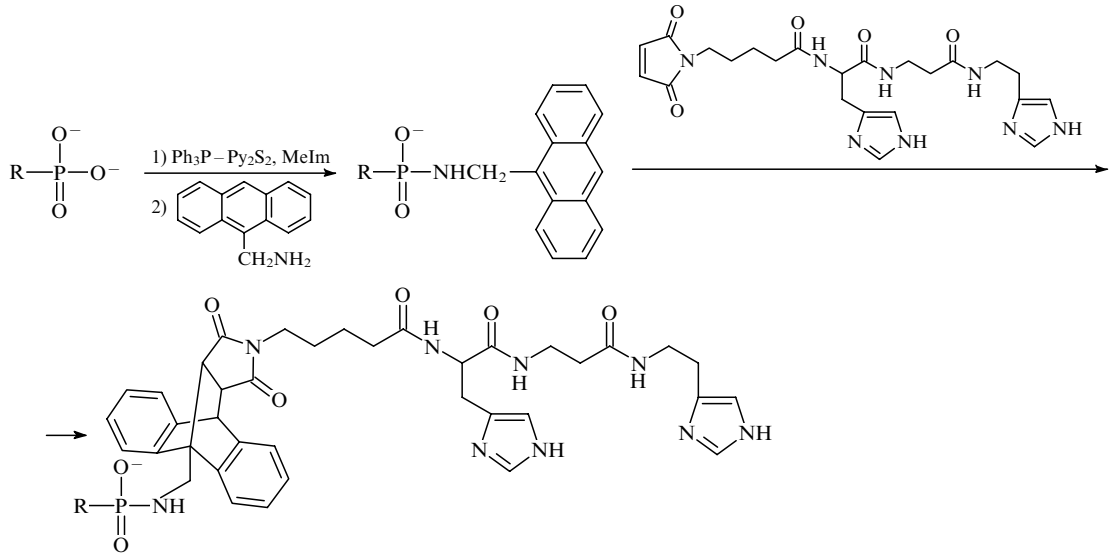


Figure 7. Metal-independent catalytic groups R used in the construction of synthetic ribonucleases based on oligonucleotide conjugates.
Sites of $tRNA^{Phe}$ cleavage with bisimidazole oligonucleotide conjugates are indicated by arrows; (*1*) oligonucleotide A, (*2*) oligonucleotide B.

Two radically different approaches to the synthesis of oligonucleotide conjugates with various ligands, *viz*., the introduction of ligands in one of the stages of the oligonucleotide synthesis and the post-synthetic modification of the oligonucleotide, are of considerable current use.[230] Several alternative procedures for performing both strategies are available. The first approach most often involves the automated synthesis of oligonucleotides based on amidophosphites of protected nucleosides and ligands. Monomeric blocks containing fragments of various metal complexes[231–235] and imidazole-containing constructions[236–240] were proposed for the preparation of synthetic ribonucleases based on oligonucleotide conjugates. The successive synthesis of the oligonucleotide and catalytic fragments of the conjugate at the same solid-phase carrier is a promising variation of this approach.[221, 241] However, this approach is unavailable for the majority of potential consumers of these conjugates because of the necessity of synthesising unique amidophosphites in each particular case and the problems associated with the choice of protective groups and procedures for their removal in the case of the successive synthesis of oligonucleotide conjugates. On the contrary, the synthesis of oligonucleotides containing functional groups along with 5′- or 3′-terminal phosphates and amino, hydroxyl and carboxyl groups was well developed by many biochemical companies due to which the post-synthetic modification of oligonucleotides shows considerable promise. Variations

Scheme 4



R is deoxyoligonucleotide; MeIm is methylimidazole.

of the functionalisation of the above-mentioned groups were considered in the studies.[230, 242] The latter approach was widely used in the synthesis of ribonucleases containing metal complexes,[103, 205, 210, 211, 229] polyamines[215, 219] or imidazole derivatives.[226] The soft procedure for activation of the terminal phosphate group[243] makes possible the preparation of oligonucleotide conjugates with molecules bearing aliphatic amino groups without protection of the imidazole[224, 225, 244] or guanidinium[222, 223] fragments. Beloglazova *et al.*[245] proposed an ingenious approach to the preparation of oligonucleotide conjugates with molecules containing virtually any functional groups necessary for the design of highly efficient catalytic centres. The authors attached the catalytic centre to the terminal phosphate group of deoxyoligonucleotide by a Diels–Alder reaction between the anthracene fragment and the double bond of a maleimide group (Scheme 4).

* * *

Intensive studies on the construction of synthetic catalysts for the specific cleavage of ribonucleic acids have resulted in the preparation of promising compounds belonging to a new family of structural probes for investigation of RNA conformations and for the design of conjugates of antisense oligonucleotides. Low-molecular-weight synthetic catalysts capable of cleaving RNA with an efficiency and selectivity close to those typical of natural ribonulceases are as yet unavailable. However in many cases, this efficiency is not necessary. In particular, in spite of low efficiency, synthetic ribonucleases as structural probes have advantages over natural enzymes. For example, when binding with RNA, ribonuclease A, which is an enzyme catalysing hydrolysis predominantly of pyrimidine–purine sites in single-stranded regions, changes the RNA structure and even leads to untwisting of double-stranded regions, which limits the possibilities of its use in structural studies. Small synthetic molecules do not destroy the structures of nucleic acids and cleave the latter only within single-stranded regions existing in the native molecule, which allows one to obtain information on the biologically active RNA structure.

Studies both aimed at examining processes occurring in the active centres of enzymes and directed towards modelling of these processes in small chemical nucleases are being continued. It is hoped that synthetic nucleases, which may be highly competitive with natural biocatalysts and even outperform them in efficiency, will be prepared in the coming years. Such compounds can help in designing a new generation of highly efficient antiviral and anticancer drugs.

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