Site-specific synthetic ribonucleases based on oligonucleotide conjugates with metal-independent organic catalysts of hydrolysis of phosphodiester bonds

*I. Yu. Garipova and V. N. Silnikov**

Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences, 8 prosp. Akad. Lavrent´eva, 630090 Novosibirsk, Russian Federation. Fax: +7 (383 2) 33 3677. E-mail: silnik@niboch.nsc.ru

The review surveys the results obtained in investigations on the design of site-specific catalysts of the phosphodiester bond cleavage in ribonucleic acids based on deoxyribonucleotide conjugates with peptide mimics.

Key words: oligonucleotides, imidazole, artificial ribonucleases, hydrolysis, RNA.

The development of the theory and procedures for the targeted action on biopolymers at the molecular and cellular levels is presently a major area of scientific investigations in bioorganic chemistry and molecular biology. Among objects subjected to targeted chemical modifications, the key biopolymers, *viz.*, nucleic acids, are of particular interest. The design of reagents which provide the selective degradation of particular elements of these biopolymers offers possibilities for the selective influence on the functioning of individual genes with the aim of correcting biochemical processes in organisms and opens the way to the selective destruction of foreign nucleic acids for the purpose of suppressing multiplication of infectious agents (bacteria, viruses, micoplasms, *etc.*). These reagents may revolutionize the strategy of treatment of virtually all diseases because they open up possibilities of influencing the deepest origin of pathological states.

The most efficient approach to the design of reagents targeted to particular nucleic acids involves the synthesis of reactive oligonucleotide derivatives. The prospects of developing rational approaches in chemotherapy based on the use of oligonucleotide derivatives gave impetus to a large body of recent research devoted to both the synthesis of such reagents and examination of their chemical and biological activities.[1—3]

There is a rather broad spectrum of organic compounds which (at high concentrations) cleave the phosphodiester bonds in RNA. The idea of designing site-specific synthetic ribonucleases by constructing deoxyoligonucleotide conjugates with organic catalysts was first conceived in the late 1980s.[4] Komiyama and Inokawa[5] were the first to report the site-specific cleavage of an RNA target with this type of oligonucleotide conjugates. A 19-mer deoxyribonucleotide complementary to the A-44—A-62 fragment of the yeast $tRNA^{Phe}$ and containing the ethylenediamine residue bound at position 5´ through the urethane linker is an example of synthetic nucleases. This deoxyribonucleotide cleaved ~10% of the RNA target at 50 °C in 4 h. Later, efforts of several research groups were directed at preparing more efficient artificial nucleases whose catalytic sites contain the imidazole or alkylamine residues (or their combinations), short peptides with alternating amino acid residues.[6—9] However, the efficiency of RNA cleavage was no higher than 10—40%.[10,11]

Simultaneously, synthetic ribonucleases were constructed based on oligonucleotide conjugates with different metal complexes. A series of synthetic ribonucleases exhibit the RNA-cleaving activities with the efficiencies close to 100%.[12] Synthetic ribonucleases based on 12-mer 2´-modified oligoribonucleotides to which rare-earth complexes are bound perform up to 40 RNA-cleavage events at 37 °C during 64 h at the reagent concentration of 1 μm L^{-1} in the presence of a 50-fold excess of RNA.[13]

Such a substantial difference in the efficiencies of conjugates based on metal complexes and organic catalysts of hydrolytic cleavage of the phosphodiester bonds is attributed to the fact that the imidazole, amino, and carboxy groups are by themselves weak catalysts of the corresponding processes. High efficiencies of natural biocatalysts based on these groups are achieved due to the optimum three-dimensional arrangement of the catalytic site. The reproduction of the geometric parameters of the catalytic sites of natural RNA-hydrolyzing enzymes appeared to be a challenging task.

At the same time, researchers face problems when using oligonucleotide conjugates based on metal complexes in biological systems. In particular, proteins and nucleic acids, which are the major components of biological systems, contain a large number of chelating sites resulting in dissociation of metal ions even from stable

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 7, pp. 1025—1030, July, 2002.

1066-5285/02/5107-1112 $27.00

complexes and, as a consequence, in nonspecific destruction of non-target biopolymers.

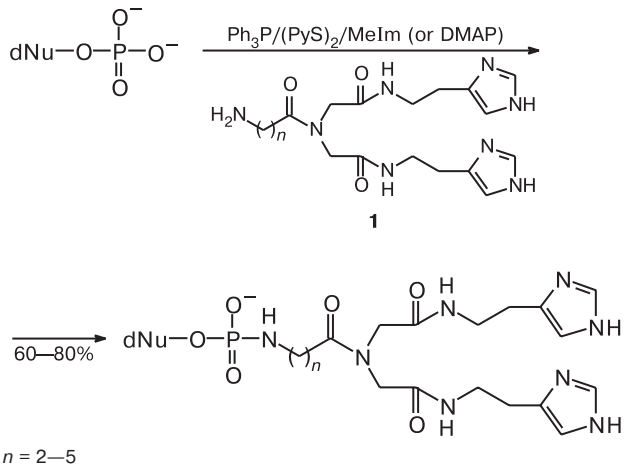
Hence, the construction of site-specific artificial nucleases based on oligonucleotide conjugates with metal-independent catalysts of RNA hydrolysis is still a topical problem.

The present review surveys the results of the design of these compounds.

Pancreatic ribonuclease A is one of the most extensively studied RNA-hydrolyzing enzymes. According to the results of different investigations, the catalytic site of this enzyme contains from 6 to 11 functionally significant amino acid residues among which His12, His119, and Lys40 are most important. Conjugates of intercalating compounds with peptide mimics containing two imidazole residues, which model the catalytic site of RNase A, efficiently cleave the phosphodiester bonds in the CpA and UpA sequences.[14]

On the basis of these data, we synthesized deoxyribooligonucleotide conjugates with iminodiacetic acid derivatives containing two imidazole residues (**1**).[15] The terminal phosphate groups in oligonucleotides were activated by the $Ph_3P/(PyS)_2$ couple in the presence of *N*-methylimidazole (MeIm) or 4-dimethylaminopyridine (DMAP). Using histamine as an example, we have demonstrated[16] that the introduction of a protective group into the imidazole ring is unnecessary, and the resulting reactive derivative reacts predominantly with an aliphatic amino group. Compound **1** was attached at the 5′- or 3′-phosphate group of deoxyribooligonucleotides through the phosphoramide bond (Scheme 1).

Scheme 1



The oligonucleotide (address) component of the conjugate was chosen so that the catalytic group is oriented toward the CpA sequences in the RNA target (Fig. 1). Optimization of the linker length made it possible to achieve the cleavage efficiency of 60—80% (5—8 h, 37 °C,

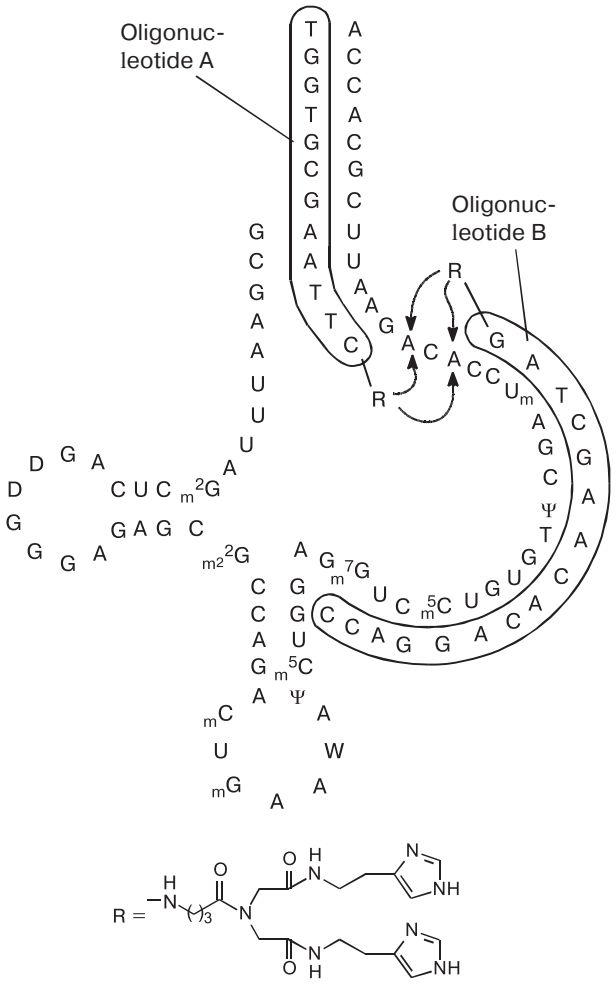


Fig. 1. Structures of yeast $tRNA^{Phe}$ and oligonucleotide conjugates A and B prepared by the attachment of the catalytic group R to 3′- (A) or 5′-phosphates (B) of deoxyribooligonucleotides. The sites of the phosphodiester bond cleavage in the RNA target are indicated by arrows.

pH 7.0) for different targets.[17,18] All other conditions being the same, the efficiencies of conjugates bearing catalytic groups at the 3′-termini of the oligonucleotides proved to be 20—30% higher than those of their 5′-analogs.

Attempts to increase the degree of hydrolysis by increasing the time of incubation of an RNA target with the artificial RNases did not give the desired results. The degree of hydrolysis reached the plateau after 5—8 h. Subsequent studies demonstrated that the phosphoramide bond that links the oligonucleotide and the catalytic components of the conjugate underwent autocatalytic hydrolysis under the experimental conditions. The hydrolysis half-life varied from 45 min to 6 h depending on the type of the terminal nucleotide and the position of the phosphate group.* One would expect that the attachment of

* Unpublished results.

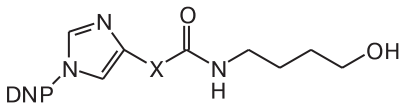
the catalytic groups proposed in the cited studies to oligonucleotides through stable linkers will enhance the efficiencies of this type of artificial nucleases.

Presently, several strategies are used for the preparation of oligonucleotide conjugates with peptides or other compounds containing functional groups. The synthesis of the corresponding phosphoramidites and their subsequent use in the conventional oligonucleotide synthesis and the postsynthetic modification of oligonucleotides are the most common methods.[19,20]

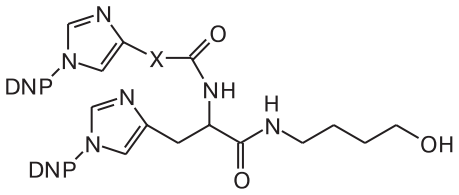
The former approach implies the design of the corresponding phosphoramidite for the synthesis of each oligonucleotide conjugate. To the contrary, the postsynthetic modification enables one to prepare series of conjugates starting from the same basic oligonucleotide.

We used both these strategies for the construction of stable synthetic ribonucleases. In particular, with the aim of eliminating the unstable phosphoramide bond, we synthesized a series of hydroxy derivatives **2a**,**b** and **3a**,**b** containing one or two protected imidazole residues. These compounds can be used for the synthesis of the corresponding phosphoramidites followed by the introduction of catalytic groups under the conditions of the conventional solid-phase oligonucleotide synthesis or, alternatively, can be directly introduced into an oligonucleotide (the triester method of synthesis).[21]

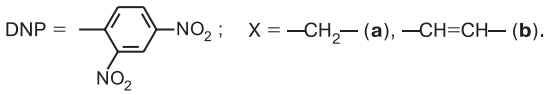
The choice of protective groups is the major problem in the construction of conjugates with the use of functionalized phosphoramidites. These groups must be compatible with the peptide synthesis (synthesis of a catalytic group), stable under the conditions of the phosphoramidite and oligonucleotide syntheses, and removable under mild conditions which do not cause conjugate



2a,**b**



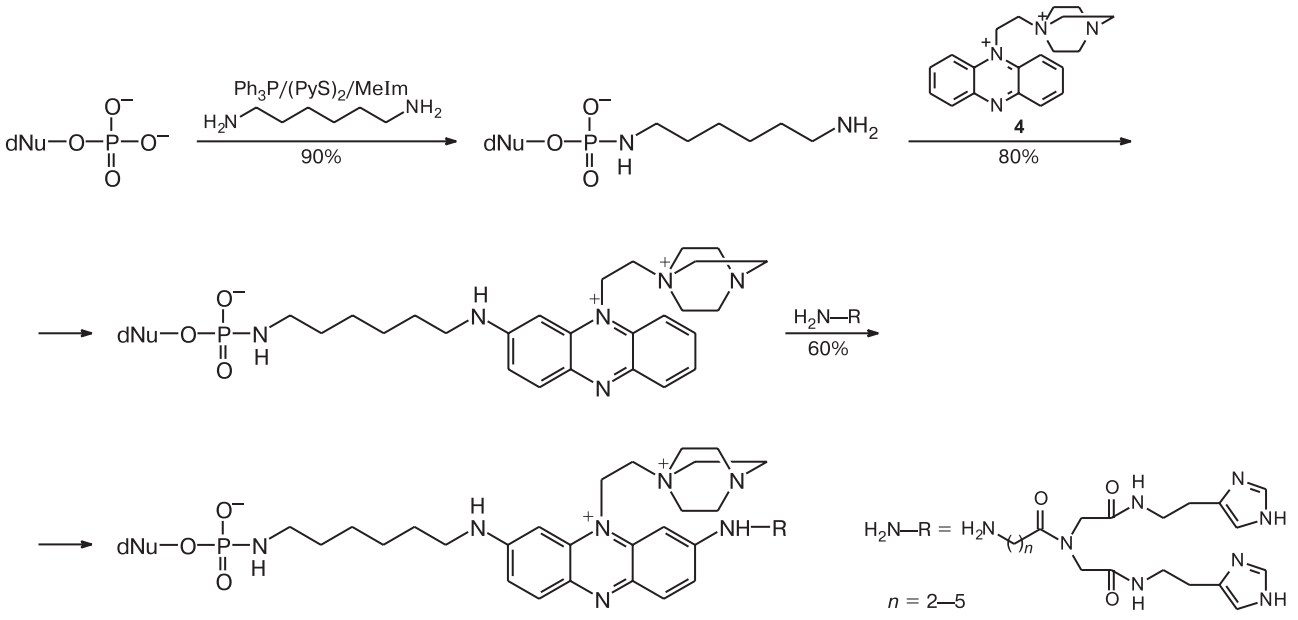
3a,**b**



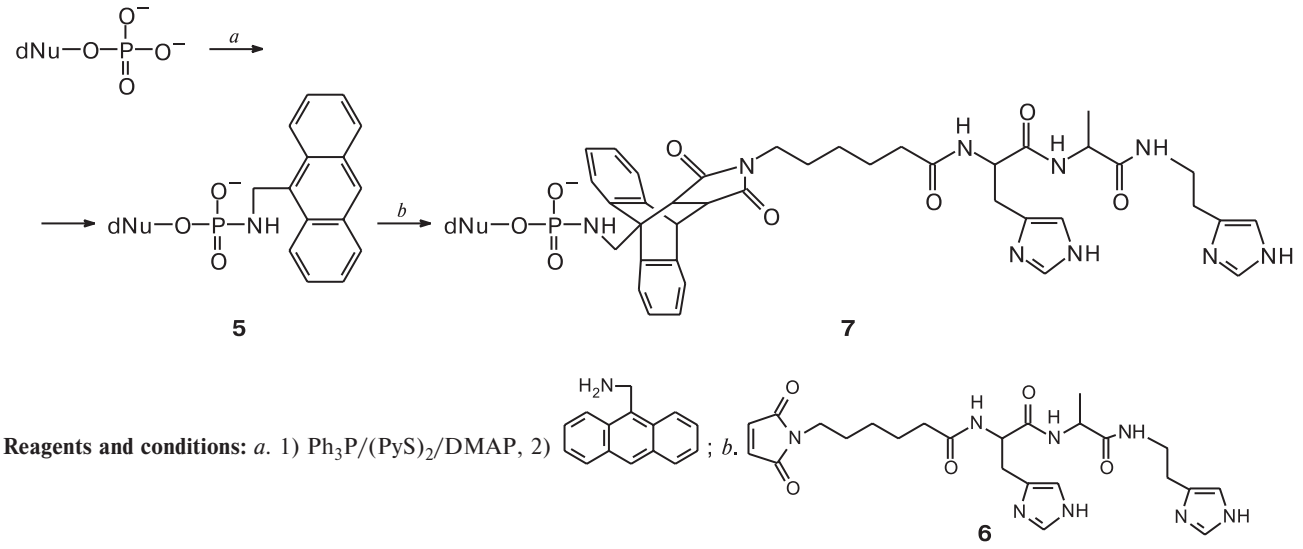
destruction. Taking into account that the preparation of the corresponding monomers is a cumbersome procedure (particularly, in the case of polyfunctional compounds), this approach to the search for the optimum structures of synthetic ribonucleases is unlikely to be efficient.

For the most part, the problem of the choice of the protective group can be solved by performing the synthesis of the catalytic and address components of the artificial nucleases separately. In order to prepare conjugates, precursor groups which allow one to attach catalytic sites under mild conditions must be introduced into the oligonucleotide in the course or after its synthesis. To realize this approach, we used the two-step proce-

Scheme 2



Scheme 3



Reagents and conditions: *a*. 1) $Ph_3P/(PyS)_2$/DMAP, 2) ; *b*.

dure for the introduction of catalytic groups into oligonucleotides with the use of specially designed derivatives of bisquaternary phenazinium salts **4** as precursors (Scheme 2).[22] We employed this method in the synthesis of a series of oligonucleotide conjugates. Unfortunately, the compounds thus obtained exhibited very low efficiencies with respect to an RNA target.* Apparently, this is associated with an inappropriate orientation of the catalytic groups bound to the oligonucleotide through a linker which behaves as an intercalator.[23]

We also used anthracene derivatives as precursor groups (Scheme 3).[24] To introduce the anthracene residue into the oligonucleotide, we initially activated the terminal phosphate group with the $Ph_3P/(PyS)_2$ couple in the presence of DMAP. This activation is reputed to work well. Treatment of the reaction mixture with 9-aminomethylanthracene afforded the corresponding anthracene derivative **5** in high yield. Cycloaddition of maleimide derivative **6** to derivative **5** in an aqueous medium gave rise to product **7** in nearly quantitative yield. At room temperature, compound **6** is poorly soluble at neutral pH. The addition of organic solvents (DMF or DMSO) to the reaction mixture increased the solubility of compound **6**, but the yield of conjugate **7** was at most 15—20%. The optimum reaction conditions involve incubation of oligonucleotide derivative **5** and compound **6** at pH 5.5—6 and 50 °C. The course of the reaction was monitored by UV spectroscopy by following the disappearance of the absorption band at 338 nm. The yield of conjugates **7** reached 90—95%, while the destruction of the oligonucleotides was no higher than 3—5%.

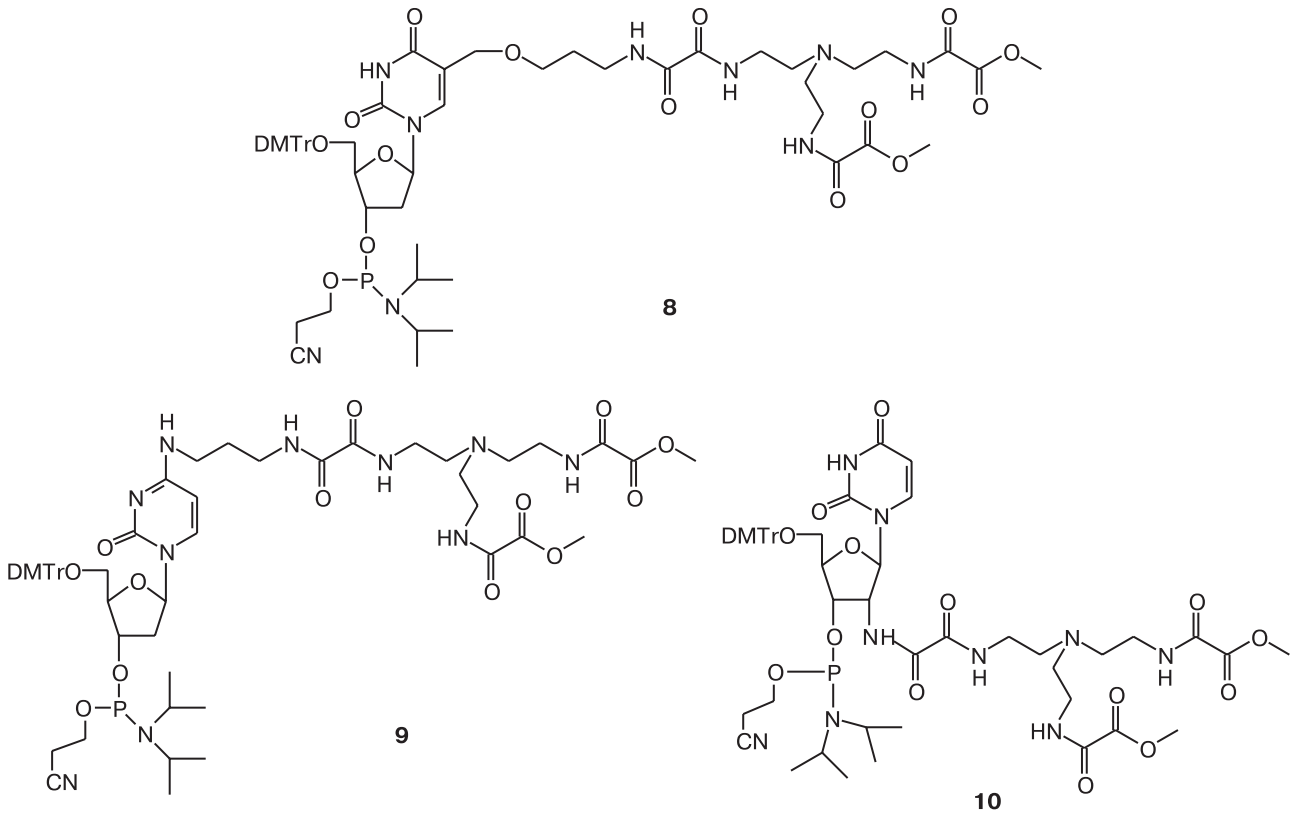
This procedure for the synthesis of artificial ribonucleases has a number of advantages. The cycloaddition product generated in the course of the reaction prevents the phosphoramide bond from intramolecular hydrolysis and stabilizes such conjugates under the conditions of hydrolysis of an RNA target. At the same time, maleimide derivative **6** used in the second step can be readily prepared by standard techniques of the peptide chemistry, which opens up considerable possibilities of optimizing the structure of the catalytic portion of such conjugates.

We prepared for the first time RNA-hydrolyzing metal-independent conjugates which perform quantitative cleavage of an RNA target at 37 °C in 20—60 min.[24] It should be noted that if the catalytic groups are oriented toward the target region deprived of the CpA sequences, it is the phosphodiester bond in the nearest pyrimidine—purine site that is subjected to hydrolysis. In the latter case, the rate of hydrolysis is decreased by a factor of 2—3.[25,26]

The time of formation of complexes of 16—18-mer oligonucleotides with structured RNAs is comparable with the time of the cleavage of an RNA target under the action of the most efficient oligonucleotide conjugates.[27] At the same time, the strengths of binding of an oligonucleotide conjugate with an RNA target and its hydrolysis products are virtually the same, which leads to the inhibition of artificial nucleases by hydrolysis products and, as a consequence, the catalytic mode of hydrolysis cannot take place. Hence, optimization of the structure of the conjugate as a whole, and not only of its catalytic portion, is of importance for the enhancement of the efficiency of RNA cleavage (a decrease in the concentration of the reagents and an increase in the hydrolysis rate).

The ways of overcoming these problems are known, these were tested for oligonucleotide conjugates with metal complexes. It was suggested that a catalytic group

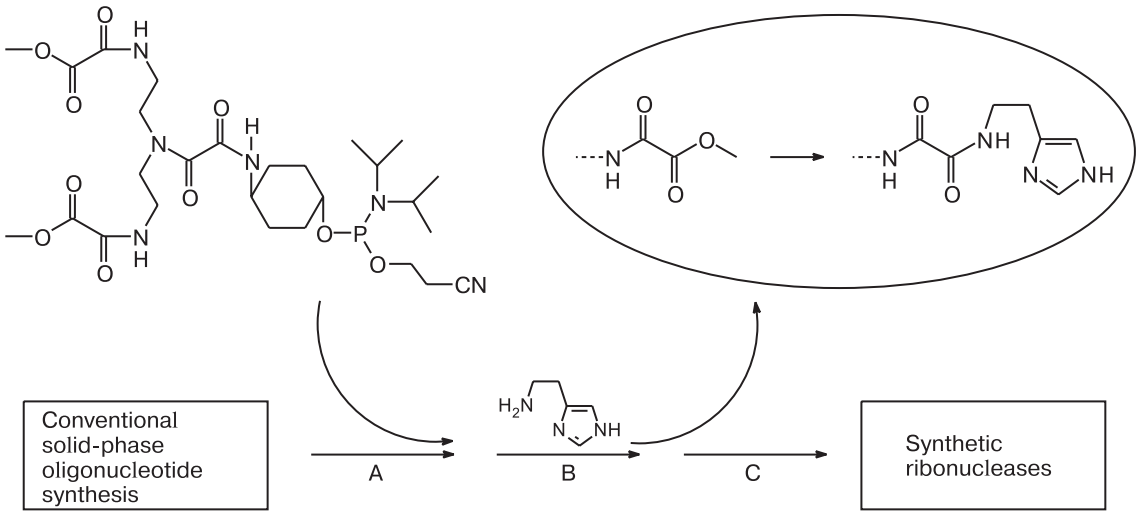
* Unpublished results.



be inserted into the middle part of an oligonucleotide to decrease the constant of binding of an oligonucleotide conjugate to the RNA target. With the aim of preventing hydrolysis in a stable double-stranded fragment of the complex, an artificial looping-out of several nucleotides units protecting the catalytic group was formed.[28]

The strategy for the insertion of catalytic groups at the termini of oligonucleotides proved to be unsuitable for the above approach. We developed a more common procedure for the design of oligonucleotide conjugates with catalytic groups. For this purpose, we synthesized a set of phosphoramidites **8**—**10** which contained less bulky

Scheme 4



A, the introduction of a monomer bearing methyl oxamate precursor groups in a particular step of the oligonucleotide synthesis; B, the reaction of protected oligonucleotide with histamine; C, deprotection and elimination of the oligonucleotide conjugate from the polymeric carrier.

precursor groups based on methyl oxamate in different positions of nucleotides. These phosphoramidites are stable in the course of the oligonucleotide synthesis and, at the same time, are readily functionalized in the postsynthetic step.

These phosphoramidites can be used for the insertion of one or several modified nucleotide units into different positions of the oligonucleotide address, which allows one to prepare oligonucleotide conjugates containing from two to several tens imidazole residues.[29] The synthesis of oligonucleotide conjugates with the use of model precursor groups based on methyl oxamate is presented in Scheme 4. Synthetic ribonucleases, which were prepared according to this scheme and contained two imidazole residues, cleaved an RNA target with the efficiency of 90—100% in 8 h.[30]

It is hoped that the proposed strategy in combination with the techniques of combinatorial chemistry will enable one to prepare synthetic ribonucleases highly competitive in efficiency with natural biocatalysts in the nearest future.

We thank Academician V. V. Vlassov and M. A. Zenkova for organizing and performing analysis of the catalytic activities of the conjugates.

This study was financially supported by the Ministry of Education of the Russian Federation (Grant in Fundamental Natural Sciences), by the Russian Academy of Sciences (Grant for Young Scientists No. 219), and by the US Civilian Research and Development Foundation (CRDF, Grant REC-008).

Reference

1. D. G. Knorre, V. V. Vlassov, V. F. Zarytova, A. V. Lebedev, and O. S. Fedorova, *Design and Targeted Reactions of Oligonucleotide Derivatives*, CRC, Boca Raton, 1994.
2. P. D. Cook, in *Antisense Research and Applications*, Eds. S. T. Crooke and B. Lebleu, CRC, Boca Raton, 1993.
3. C. Helene and J.-J. Tulme, *Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression*, Ed. J. S. Cohen, Macmillan Press, London, 1989.
4. C. H. Stein and J. S. Cohen, *Cancer Res.*, 1988, **48**, 2659.
5. M. Komiyama and T. Inokawa, *J. Biochem.*, 1994, **116**, 719.
6. M. A. Reynolds, T. A. Beck, P. B. Say, D. A. Schwartz, B. P. Dwyer, W. J. Daily, M. M. Vaghefi, M. D. Metzler, R. E. Klem, Jr., and L. J. Arnold, *Nucleic Acids Res.*, 1996, **24**, 760.
7. J. Hovinen, A. Guzaev, E. Azhayeva, A. Azhaev, and H. Lönberg, *J. Org. Chem.*, 1995, **60**, 2205.
8. D. V. Pyshnyi, M. N. Repkova, S. G. Lokhov, E. M. Ivanova, A. G. Ven´yaminova, and V. F. Zarytova, *Bioorg. Khim.*, 1997, **23**, 497 [*Russ. J. Bioorg. Chem.*, 1997, **23** (Engl. Transl.)].
9. K. Ushijima, H. Gouzu, K. Hosono, M. Shirakawa, K. Kogosima, K. Takai, and H. Takaku, *Biochim. Biophys. Acta*, 1998, **1379**, 217.
10. B. N. Trawick, A. T. Daniher, and J. K. Bashkin, *Chem. Rev.*, 1998, **98**, 939.
11. V. N. Sil´nikov and V. V. Vlassov, *Usp. Khim.*, 2001, **70**, 562 [*Russ. Chem. Rev.*, 2001, **70** (Engl. Transl.)].
12. J. Hall, D. Husken, and R. Häner, *Nucleic Acids Res.*, 1996, **24**, 3522.
13. R. Häner, J. Hall, A. Pfützer, and D. Hüsken, *Pure Appl. Chem.*, 1998, **70**, 111.
14. M. A. Podyminogin, V. V. Vlassov, and R. Giege, *Nucleic Acids Res.*, 1993, **21**, 5950.
15. V. Silnikov, G. Zuber, J.-P. Behr, R. Giege, and V. Vlassov, *Phosphorus Sulfur Silicon*, 1996, **109—110**, 277.
16. D. G. Knorre, P. V. Alekseev, Y. V. Gerassimova, V. N. Silnikov, G. A. Maksakova, and T. S. Godovikova, *Nucleosides Nucleotides*, 1998, **17**, 397.
17. V. Vlassov, T. Abramova, T. Godovikova, R. Giege, and V. Silnikov, *Antisense Res. Dev.*, 1997, **7**, 39.
18. L. Yurchenko, V. Silnikov, T. Godovikova, G. Shishkin, J.-J. Toulme, and V. Vlassov, *Nucleosides Nucleotides*, 1997, **16**, 1721.
19. D. A. Stetsenko, A. A. Arzumanova, V. A. Korshun, and M. D. Geit, *Mol. Biol.*, 2000, **34**, 998 [*Russ. J. Mol. Biol.*, 2000, **34** (Engl. Transl.)].
20. A. S. Butorin, G. N. Grimm, and K. Elen, *Mol. Biol.*, 2000, **34**, 946 [*Russ. J. Mol. Biol.*, 2000, **34** (Engl. Transl.)].
21. A. Yu. Karyagin, T. V. Abramova, V. N. Sil´nikov, and G. V. Shishkin, *Izv. Akad. Nauk, Ser. Khim.*, 2000, 536 [*Russ. Chem. Bull., Int. Ed.*, 2000, **49**, 540].
22. V. N. Sil´nikov, N. P. Luk´yanchuk, and G. V. Shishkin, *Izv. Akad. Nauk, Ser. Khim.*, 1999, 1157 [*Russ. Chem. Bull.*, 1999, **48**, 1146 (Engl. Transl.)].
23. D. V. Pyshnyi, S. G. Lokhov, V. N. Sil´nikov, G. V. Shishkin, E. M. Ivanova, and V. F. Zarytova, *Bioorg. Khim.*, 1999, **25**, 40 [*Russ. J. Bioorg. Chem.*, 1999, **25** (Engl. Transl.)].
24. N. G. Beloglazova, V. N. Sil´nikov, M. A. Zenkova, and V. V. Vlassov, *FEBS Lett.*, 2000, **481**, 277.
25. N. Beloglazova, V. Silnikov, M. Zenkova, and V. Vlassov, *Abstr. of Papers of Int. Conf. "RNA as Therapeutic and Genomics Target" (Novosibirsk, 30 August—2 September 2001)*, Novosibirsk, 2001, P-14.
26. N. G. Beloglazova, A. Yu. Epanchintsev, V. N. Sil´nikov, M. A. Zenkova, and V. V. Vlassov, *Mol. Biol.*, 2001 [*Russ. J. Mol. Biol.*, 2001, **35** (Engl. Transl.)].
27. V. A. Petyuk, R. Giege, V. V. Vlassov, and M. A. Zenkova, *Mol. Biol.*, 2000, **34**, 879 [*Russ. J. Mol. Biol.*, 2000, **34** (Engl. Transl.)].
28. D. Huesken, G. Goodall, M. J. J. Blommers, W. Jahnke, J. Hall, R. Haener, and H. E. Moser, *Biochemistry*, 1996, **35**, 16591.
29. N. N. Polushin, *Collect. Czech. Chem. Commun., Coll. Symp. Ser.*, 1999, **2**, 145.
30. N. G. Beloglazova, N. N. Polushin, V. N. Sil´nikov, M. A. Zenkova, and V. V. Vlassov, *Dokl. Akad. Nauk,* 1999, **369**, 827 [*Dokl.*, 1999 (Engl. Transl.)].

Received October 12, 2001;
in revised form February 13, 2002