Ukrainica Bioorganica Acta 1 (2007) 11—19

Ukrainica Bioorganica Acta

www.bioorganica.org.ua

Metal complexes of 1,4,7-triazacyclononane and their oligonucleotide conjugates as chemical nucleases

I.Ya. Dubey*, L.V. Dubey, E.V. Piletska[1], S.A. Piletsky[1]

Institute of Molecular Biology and Genetics, NAS of Ukraine
150 Zabolotny Str., Kyiv, 03143, Ukraine

[1] *Cranfield Health, Cranfield University*
Silsoe, Bedfordshire, MK45 4DT, UK

Summary. The efficiency of nucleic acids cleavage by complexes of 1,4,7-triazacyclononane (TACN) with various transition metals under physiological conditions was studied. It was found that copper (II), europium (III) and terbium (III) macrocyclic complexes cleaved ApA diribonucleotide with significant activity, but complexes of the other studied metal ions (Co^{2+}, Fe^{3+}, Mn^{2+}, V^{3+} and Zn^{2+}) did not show nucleolytic activity. TACN coomplexes with all studied metals except zinc demonstrated nuclease activity on double-stranded pBR322 plasmid DNA. The efficient DNA cleavage was promoted by lanthanide complexes that probably work via hydrolytic rather than oxidative mechanism, whereas complexes of Red-Ox active metals (especially iron, copper and vanadium) could catalyze DNA oxidative scission. Vanadium complex was found to be one of the most active DNA cleavers. Model T_{15} deoxyoligonucleotide-TACN conjugate was obtained by post-synthetic coupling of N-carboxyalkyl derivative of bis(N-trifluoroacetyl)-protected azamacrocycle with 5'-aminoalkyl-functionalized oligonucleotide. Europium complex of T_{15}-linked TACN demonstrated the ability to cleave a complementary polyadenylic acid.

Keywords: chemical nucleases, metallocomplexes, triazacyclononane, nucleic acids cleavage, oligonucleotide conjugates.

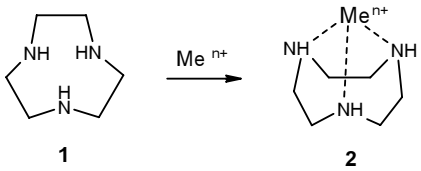
Introduction. Biomimetic hydrolysis of DNA and RNA is of increasing importance in biotechnology and medicine. The ability to cleave nucleic acids efficiently and selectively will offer many practical applications including gene manipulations, the design of structural probes and the development of novel therapeutics.

Biological systems have developed nuclease and topoisomerase enzymes to catalyze DNA strand cleavage and repair. Many of these hydrolytic enzymes are metalloenzymes, utilizing hard Lewis acids such as Ca(II), Mg(II) and Zn(II) to activate both the P-O bond and the water nucleophile. The application of synthetic metal chelating compounds in DNA and RNA hydrolysis has attracted considerable attention (see reviews [1-8]).

Nucleic acids cleavage by artificial nucleases generally proceeds via hydrolytic or oxidative mechanism. Much of the earlier literature on phosphodiester degradation by catalysts such as Fe-EDTA has focused on oxidative degradation of DNA, which requires Red-Ox active metals like Cu(II), Fe(III), Mn(III) *etc* and activation by oxidant (or light). RNA can be degraded by hydrolytic P-O bond cleavage relatively easily. The hydrolytic cleavage of DNA is however challenging because of the stability of its phosphate ester bond. Unlike RNA, whose 2'-hydroxyl group serves as an internal nucleophile to promote backbone strand scission, DNA is extremely resistant to hydrolysis under physiological conditions.

*** Corresponding author.**
Tel./fax: +38044-5265598
E-mail address: dubey@imbg.org.ua

Scheme 1
*Structure of TACN (**1**) and its metallocomplexes (**2**)*



The physico-chemical properties and applications of macrocyclic metal complexes have been intensively studied. A number of macrocycles have been used as ligands for metal-promoted nucleic acids cleavage, including aza-crowns like 1,4,7-triazacyclononane (abbreviation [9]aneN$_3$ or TACN, Scheme 1). 1,4,7-Triazacyclonane-Cu(II) and 1,4,7,10-tetrazacyclododecane-Eu(III) type of compounds have been reported to hydrolyse nucleotides. Morrow *et al.* published a series of papers on using metal macrocyclic complexes including lanthanum (III), europium (III), lutetium (III), to cleave RNAs and achieve phosphate diester transesterification. Burstyn *et al.* reported that Cu([9]aneN3)Cl$_2$ is capable of cleaving activated phosphodiester and both single-stranded and double stranded DNA [9, 10].

Blake and Hay *et al.* reported details of the derivatisation of aza macrocycles and the mechanism of the hydrolysis of phosphate triesters using [Cu-([9]aneN$_3$)(OH$_2$)$_2$]$^{2+}$ as a catalyst [11, 12]. Also it was found that dinuclear copper(II) complexes of triazacyclononane accelerate the hydrolysis of 5'-capped RNAs [13] and ApA dinucleotide [14]. It was shown that phosphate diester hydrolysis occurred via an intramolecular nucleophilic attack of a metal-co-ordinated hydroxide at the phosphate centre. The rate constant is in the range of 10^{-5} s^{-1} to 10^{-7} s^{-1}. Other complexes of Red-Ox active metals (Co(III), Fe(III), Cu(II) *etc*) with ligands of more complicated structuere including dinuclear bis-TACN complexes and complexes where TACN is one of ligands were shown to be efficient DNA cleavers [15, 16].

Lanthanide ions, as hard Lewis acids with high coordination numbers and no associated Red-Ox chemistry, are well suited to mimic the reactivity of biological metal cofactors. Lanthanide ions are remarkably effective catalysts for the hydrolytic cleavage of phosphate ester bonds, including the robust bonds of DNA [17-20].

In the present work, TACN complexes with various transition metal cations were studied for their catalytic efficiency in RNA and DNA cleavage.

Results and discussion.

ApA cleavage. Experinents on the cleavage of diribonucleotide ApA by TACN complexes were carried out in the presence of following transition metal cations: Co(II), Cu(II), Eu(III), Fe(III), Mn(II), Tb(III), V(III), V(IV) and Zn(II), at pH 6.0 and 7.4 (HEPES buffer). Metal complexes were prepared *in situ* by mixing equimolar amounts of macrocycle and metal salt aqueous solutions. Cleavage reactions were monitored by HPLC, products were identified by retention times and by co-injection with authentic samples (ApA, adenosine, adenosine phosphates). Typical HPLC profile is shown in Figure 1. Cleavage reaction yields were calculated from HPLC integration data by comparison starting dinucleotide and adenosine product peak areas and taking into

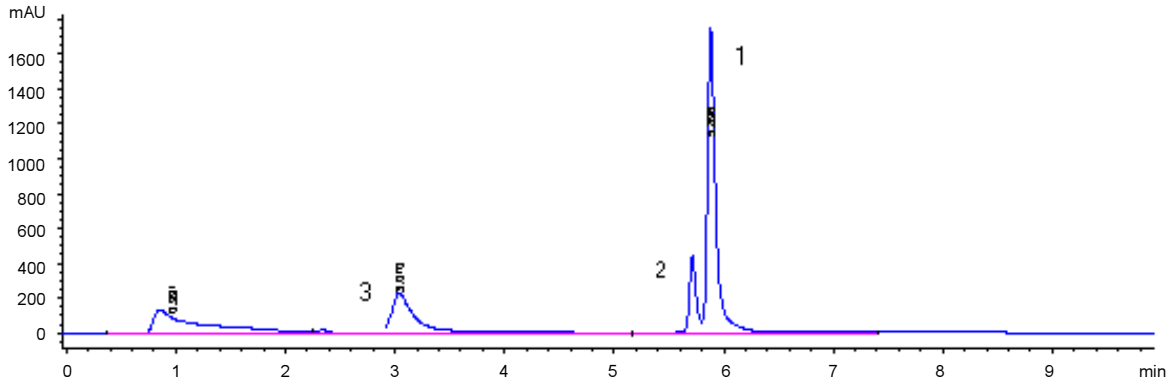


Figure 1. Reverse phase HPLC profile of ApA cleavage by TACN complex with Eu^{3+} (pH 7.4, 22 °C, 48 h). Peak 1 — ApA, 2 — adenosine, 3 — adenosine 3'-phosphate.

account that extinction coefficient ε(A)=1/2ε(ApA). TACN alone in the absence of any metal, as well as metal salts without the addition of macrocyclic ligand, were not active towards ApA dinucleotide producing virtually no cleavage. Only copper, europium and terbium were nucleolytically active in the order Cu(II)<<Tb(III)<Eu(III) (Table 1). The most reactive metal ion was Eu^{3+} whose complex hydrolysed 33 % of ApA at ambient temperature in 48 h at pH 7.4. Other metal complexes did not show any detectable cleavage at all. It is evident from the HPLC data that the ApA cleavage reaction occurs via hydrolytic mechanism producing adenosine and adenosine 3'-phosphate as main products. Cleavage reaction rate is higher at basic pH (7.4) than in acidic medium (pH 6.0). Of course, reactions at elevated temperature were faster. For example, at pH 7.4 and 40 °C reaction for Eu and Tb reached comparable yield more than 2.5 times faster. Thus, only TACN complexes with lanthanides Eu^{3+} and, to a lesser extent, Tb^{3+}, possess significant nuclease activity, whereas Cu^{2+} efficiency is lower. Visible light irradiation of the reaction mixtures did not result in the increase of the cleavage reaction yield for any metal, even those absorbing in visible region (Co, Cu, Fe).

pBR322 plasmid cleavage. Supercoiled plasmid pBR322 cleavage experiments were performed with TACN metal complexes prepared *in situ* prior to plasmid addition. The metal ion and ligand solutions were pre-mixed and then added to the DNA solution. The reactions were carried out at ambient temperature in 50 mM HEPES buffer, pH 7.4. Reaction mixtures were analyzed by agarose gel electrophoresis. Gels were stained with ethidium bromide and quantified by fluorescence imaging. The products of the supercoiled DNA (Form I) cleavage are nicked circular DNA (Form II) as a product of single-strand scission, and linear DNA (Form III) resulting from double-strand cleavage. A single cut on a strand of supercoiled DNA relaxes the supercoiling and leads to form II. A second cut on the complementary strand, in close proximity to the original cut site, linearizes the DNA to form III. Therefore, a chemical nuclease must cut the DNA at least twice to convert it from form I DNA to form III DNA. To calculate the content of various DNA forms in the reaction mixture, the observed amount of the Form I DNA was multiplied by a factor of 1.47 to account for reduced dye intercalation into supercoiled DNA [21].

Table 1

The yield of ApA cleavage by TACN complexes with some metals

Metal	Cleavage reaction yield, %			
	22 °C, 48 h		40 °C, 18 h	
	pH 6.0	pH 7.4	pH 6.0	pH 7.4
Cu(II)	6	9	7	10
Eu(III)	20	33	25	28
Tb(III)	8	22	14	20

The sample of pBR322 plasmid used in experiments contained 76-77 % of the supercoiled DNA and 23-24 % of the nicked form (which is close to 80 % of the supercoiled DNA as declared by plasmid supplier, Sigma). Preliminary experiments demonstrated that double-stranded plasmid was not cleaved by 0.5-1 mM TACN alone, in the absence of metal salts.

Cleavage experiments were carried out with 0.1 and 0.5 mM TACN complexes with the same transition metals that were studied on RNA cleavage. Figure 2 shows the products of the cleavage reaction of 0.5 mM TACN metal complexes with pBR322 plasmid. The nuclease activity of complexes decreases in the order Fe>Cu>V>Co >Mn>Tb~Eu>>Zn. In contrast to RNA cleavage, Eu and Tb ions were found to be less efficient against DNA than Cu(II), Fe(III) and vanadium (III). Copper and iron induced complete cleavage of the supercoiled DNA. Iron complex was the most active reagent and produced 17 % of the linear DNA form (7 % for Cu). It was interesting to observe high activity of V^{3+} ion as there is only limited literature on nucleic acids cleavage sby vanadium complexes [22]. In this case, 13 % of starting plasmid remained in the reaction mixture, and at the same time 7 % of the linear DNA was formed. In general, vanadium (III) was almost as active as copper (II).

The ability of TACN-metal complexes to perform double-strand DNA cleavage is in principle quite unexpected, since relatively few small molecules are known to carry out such cleavage. Usually linear DNA formation results from the intercalating of the reagent into double stranded DNA. Since the structure of TACN-metal complex makes its intercalative binding hardly

Figure 2. Gel electrophoresis of the reaction mixtures obtained upon the treatment of pBR322 plasmid (50 mM HEPES, pH 7.4, ambient temperature, 15 h) by 0.5 mM TACN complexes with metals: Co (lane 1), Cu (lane 2), Eu (lane 3), Fe (lane 4), Mn (lane 5), Tb (lane 6), V (lane 7) and Zn (lane 8). Lane 9 — control (plasmid in 0.5 mM TACN, 50 mM HEPES, no metal).

probable, their activity can be assisted by the enhanced affinity of the positively charged complex for the negatively charged DNA phosphodiester backbone [23].

Whereas substantial cleavage was observed with 0.5 mM TACN-metal complexes, none of 0.1 mM metal complexes demonstrated significant activity. The content of the Form II DNA varied from 25 % for vanadium to 32 % for iron (with 24 % of the Form II present in the starting plasmid solution). Manganese and zinc demonstrated zero activity. Thus only few percent increase of the nicked DNA was achieved by 0.1 mM TACN-metal complexes. The results on the content of cleavage products in the reaction mixtures are presented in Table 2.

We have observed nucleic acids cleavage by metalocomplex that did not require external activating agent to produce DNA cleavage, in contrast to numerous metal complexes reported in the literature that require exogeneous oxidant to induce strand scission. However, all cleavage reactions were performed in «normal» aqueous buffers, i.e. they might contain dissolved oxygen. Reaction mechanism, most probably, is based on oxidative DNA cleavage, although there are reports in the literature proposing hydrolytic rather than oxidative cleavage mechanism for some metal complexes, espeially those of lanthanides [23]. Cu-TACN complex was previously shown to be capable of cleaving both single-stranded and double-stranded DNA under both aerobic and anaerobic conditions and via different mechanisms, although an efficiency of single-stranded DNA cleavage was rather low [24].

A hydrolytic cleavage mechanism is desirable for several reasons. Oxidative cleavage of DNA and RNA produces diffusible free radicals. For molecular biology applications, radical abstraction results in strand ends that cannot be enzymatically religated. For clinical applications, oxidative cleavage can cause indiscriminant peripheral damage to the cell, and radical diffusion may significantly hinder the specificity of cleavage that can be achieved. There has been a great deal of interest and success in designing transition-metal-based nucleases to promote DNA hydrolysis. There is an open field for the development of lanthanide-based nucleases.

The oxidative cleavage reaction could be accelerated by addition of oxidant like hydrogen peroxide. However, reactions of TACN complexes in the presence of oxidants were not studied in the present work. No efforts has been made as well to perform DNA cleavage under anaerobic conditions or in the presence of radical scavengers (DMSO, methanol). So experiments to study cleavage reaction mechanisms and identify the products still have to be performed.

Table 2

Content of three plasmid forms in the reaction mixtures obtained after the treatment of pBR322 plasmid with TACN-metal complexes of various concentrations (0.5 and 0.1 mM)

	Cu		Eu		Tb		Fe		Mn		V		Zn		Ctr
	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.5 mM	0.1 mM	
Form I	0	72	59	74	55	73	0	68	40	76	13	75	74	77	77
Form II	93	28	41	26	45	27	83	32	60	24	80	25	26	23	23
Form III	7	0	0	0	0	0	17	0	0	0	7	0	0	0	0

Reaction conditions: 50 mM HEPES, pH 7.4, ambient temperature, 15 h. Ctr — control (plasmid in 50 mM HEPES, 0.5 mM TACN, no metal, 15 h).

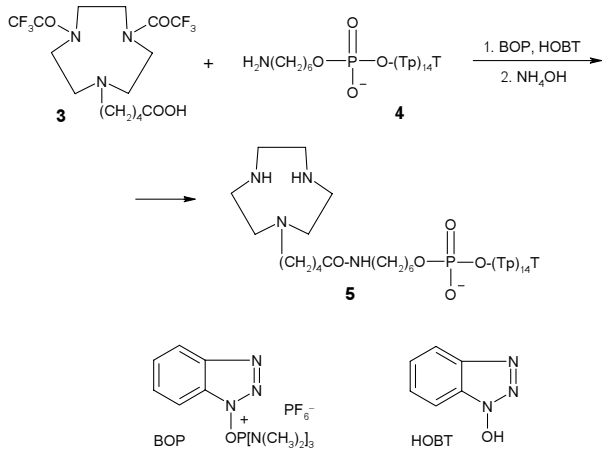
Synthesis of TACN oligonucleotide conjugate. Organometallic complexes when attached to a recognition agent such as an antisense oligonucleotide can catalyze the sequence-specific cleavage of DNA or RNA. There are numerous reports in the literature on the covalent immobilization of artificial nucleases to oligonucleotides to obtain the site-specific cleavage of the target nucleic acids [1-8, 25]. However, until recently there were no reports on the preparation of oligonucleotide conjugates with azamacrocycles. Two papers by Lonnberg *et al*. have been published in 2004 on the synthesis and RNA cleaving properties of triazadodecane macrocycle conjugate with 2'-O-methyl oligoribonucleotides [26, 27]. Zn, Cu and Ni complexes of the attached ligand have been studied and demonstrated the ability of selective cleavage of complementary RNA sequences. Triazacyclododecane ligand was attached to oligonucleotides via the linker connected to C-3 position of the azacrown. The multi-step synthesis of the reagent was complicated and laborious. We have decided to prepare TACN derivative for the conjugation with linker group attached to nitrogen atom of TACN. The idea was that N-alkyl substitution that leaves electron pair on nitrogen free would not have negative effect on the complex formation, in contrast to N-acyl modification.

To introduce a TACN residue into oligonucleotide, the preparation of derivative bearing a suitably functionalized pendant arm was necessary. Selective functionalization of the otherwise undifferentiated amino groups in azacrowns is quite complicated [28-32]. TACN reagent **3** was synthesized by the treatment of TACN with ethyl trifluoroacetate to get di-N-protected macrocycle [33] which was subsequently N-alkylated (synthetic details will be published elsewhere).

TACN macrocyclic ligand was linked to the model oligonucleotide, pentadecathymidylate, via the linker group (Scheme 2). 5'-Aminoalkyl functionalized deoxyoligonucleotide T_{15} (**4**) was used for the reaction with COOH-containing reagent **3**. The coupling reaction was performed in the presence of phosphonium activating agent BOP and 1-hydroxybenzotriazole (HOBT) catalyst, according to the previously published procedure [34]. BOP-HOBT system is a highly efficient coupling reagent for the preparation of

Scheme 2

Synthesis of oligonucleotide conjugate with TACN



oligonucleotide conjugates (see e.g. [35] for references). Reaction was monitred by HPLC and was complete in 4 h. Crude product was treated with concentrated ammonia overnight to remove N-trifluoroacetyl protecting groups from the macrocycle, and the final conjugate **5** was purified by polyacrylamide gel electrophoresis. The conjugate migrated in the gel slower than starting 5'-aminoalkyl oligomer.

TACN-oligonucleotide conjugate as a chemical nuclease. The cleavage activity of Cu(II) and Eu(III) complexes of TACN linked to the T_{15} sequence was studied on the polyadenylic acid target. Oligonucleotide conjugate **5** was incubated with poly-A in the presence of copper and europium salts. TACN conjugate concentration in the reaction mixture was 32 μM, and 50 % excess of metal ions was used. 1.2 A_{260} of TACN-T_{15} conjugate **5** reacted with 15 A_{260} of polyadenylic acid. Taking into account extinction coefficients at 260 nm for thymidine and adenosine (8.7×10^3 and 15.4×10^3, respectively [36]), we can assume that the thymidine:adenosine ratio in the mixture was approximately 1:7, or about one T_{15} molecule per 100 adenosine residues. Since the average molecular weitht of poly-A we used was about 100 kDa, i.e. poly-A was approximately 300 bases long, up to 3 TACN-T_{15} conjugate molecules could bind to poly-A molecule in average. To quench the cleavage reaction, EDTA was added to the mixture and its aliquots were analysed by reverse phase HPLC (Figure 3).

Although poly-A is rather heterogeneos mixture of oligomers, cleavage processes can be

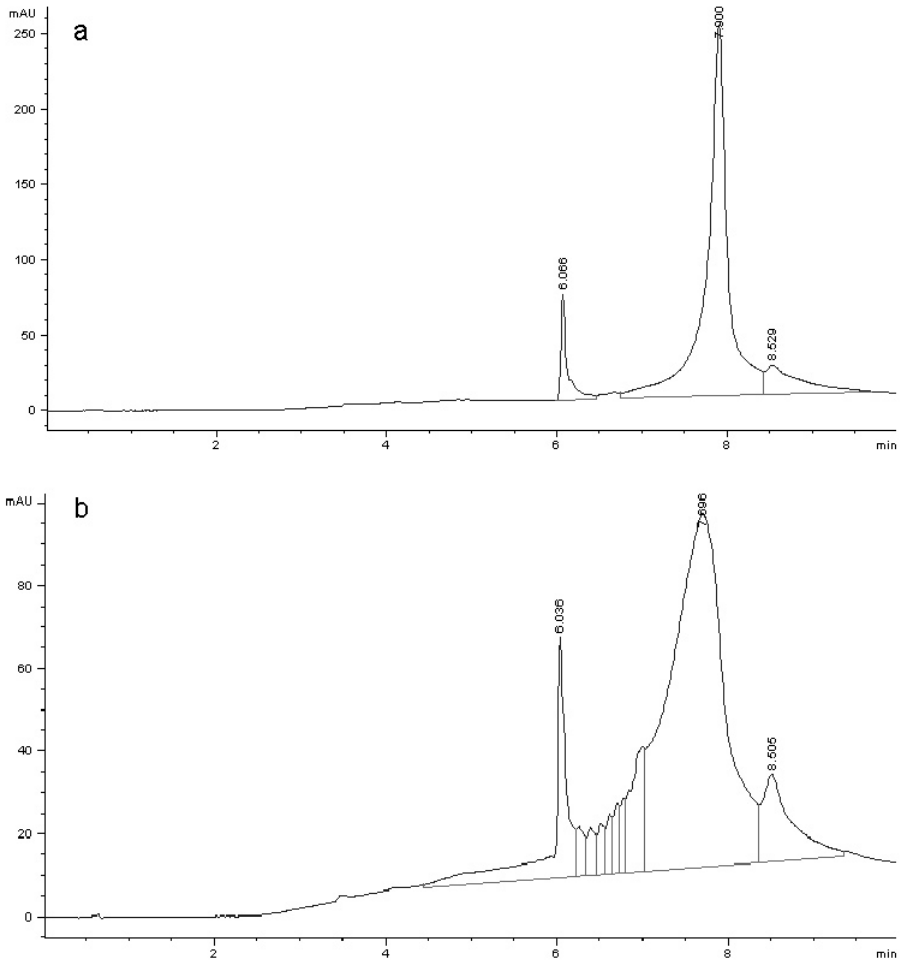


*Figure 3. Reverse phase HPLC profiles of the mixture of T_{15}-TACN conjugate **5** (retention time 6.1 min) and poly-A (retention time 7.9 min) before (a) and after (b) the incubation in the presence of europium(III) ions.*

nevertheless monitored by HPLC. As for ApA cleavage, europium complex was much more active than copper-TACN complex. It was found that almost no noticeable change of poly-A was observed in the case of Cu^{2+} complex. But Eu^{3+} complex of TACN-oligonucleotide was hydrolytically active. The treatment of poly-A with this complex resulted in the significant changes of poly-A chromatographic profile: broadening of the peak and formation of shorter oligomers. It is difficult to perform an accurate quantification of the chromatogram due to the heterogeneous character of target RNA and cleavage products. However, comparing integrated areas of the peaks in the regions 6.2-7.1 and 7.1-8.4 min led to the conclusiom that the degradation yield could be minimum 30 %. No significant T_{15} degradation was observed, so in the DNA-RNA duplex mainly RNA chain was degraded. It is in agreement with results of [26, 27] where RNA target was treated with azacrown complex linked to 2'-O-methyloligoribonucleotide which is DNA analog since it lacks 2'-hydroxy groups of RNA accelerating the hydrolysis.

Nevertheless, the cleavage reaction under the described conditions was not complete, and for the quantitative scission of target ribonucleic acid either higher excess of the conjugate or higher temperature is needed.

Thus, we have studied the activity of complexes of macrocyclic ligand TACN with various transition metals as chemical nucleases for DNA and RNA targets. It was found that RNA is cleaved most efficiently by lanthanide complexes, whereas duoble-stranded DNA is more efficiently cleaved with Red-Ox active metals — iron(III), copper(II) and vanadium(III). Zinc(II) complex was not active with nucleic acids. Reagent for the introduction of TACN ligand into oigonucleotide sequences was used to prepare TACN conjugate with T_{15} deoxyoligonucleotide. In the presence of Eu^{3+} ions (but not

with Cu^{2+}) this conjugate in rather low concentration was able to hydrolyse complementary poly-A ribonucleic acid.

Experimental part. Benzotriazol-1-yloxytris-(dimethylaminophosphonium hexafluorophosphate (BOP), anhydrous 1-hydroxybenzotriazole (HOBT), 1,4,7-triazcyclononane (TACN), triethylamine and all solvents were purchased from Acros Organics (Belgium). Agarose, boric acid, Bromophenol Blue, ethylenediaminetetraacetic acid (EDTA), ethidium bromide, glycerol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), urea and tris(hydroxymethyl)aminomethane (tris) for electrophoresis were from Sigma. Supercoiled plasmid pBR322, adenylyl(3'-5')adenosine dinucleotide (ApA), adenosine, adenosine 2'- and 3'-phosphates and 2',3'-cyclophosphate were purchased from Sigma as well. Polyadenylic acid (100 kDa) was obtained from Reanal (Hungary). Inorganic metal salts were obtained from Acros. Pentadecathymidylate T_{15} and its 5'-aminohexyl derivative were synthesised by Sigma-Genosys UK. Reverse phase HPLC was performed on Agilent 1100 Series chromatographic system (Agilent Technologies) Agarose electrophoresis gels were visualised and quantified at Syngene Gel Documentation system (Syngene Ltd, UK) and quantified using GeneTools software. For T_{15}, ε_{260} calculated according to [36] was 1.22×10^5. Reported cleavage data represent results of at least two experiments, with standard deviation below 5 %.

Nucleic acids cleavage by TACN complexes in the solution.

a) ApA cleavage. 1 mg/ml ApA solution in water (ca. 2 mM) was used for experiments. 10 mM aqueous solutions of metal salts were prepared using $CoCl_2$, $CuSO_4$, $EuCl_3$, $FeCl_3$, $MnCl_2$, VCl_3, $ZnCl_2$, and Tb(III) trifluoromethanesulfonate. TACN-metal complexes were prepared *in situ* and used without isolation. 20 μl of metal salt solution was mixed with 20 μl of 10 mM TACN solution in 100 mM HEPES buffer (pH 6.0 or 7.4), and vortexed briefly. 5 min later 20 μl of ApA solution was added and the reaction mixture vortexed again for few seconds. The reactions were performed at ambient temperature or at 40 °C, in the dark or with irradiation with visible light (100 W lamp). 5-Fold excess of TACN-metal complex was used for ApA cleavage. Final concentrations were: 0.66 mM ApA, 3.33 mM TACN, 3.33 mM metal, 33.3 mM HEPES. To quench the reaction, 50 mM EDTA (5 eq) in HEPES buffer was added to the aliquot which was then analyzed by reverse phase HPLC using Luna C18 column (3x150 mm, particle size 3 μm, Phenomenex). Analysis was performed in the gradient 0-10 % CH_3CN in 50 mM ammonium acetate, pH 6.8 (10 min, flow rate 0.3 ml/min, detection at 260 nm). Reaction yields were obtained from HPLC integration data.

b) Plasmid cleavage. For cleavage experiments, aqueous solution of plasmid pBR322 (50 μg/ml) was used. Reactions were performed in 50 mM HEPES buffer (pH 7.4) in the presence of 0.1 or 0.5 mM TACN-metal complex. Complexes were prepared *in situ* prior to use by mixing equal volumes of 10 mM TACN solution in 100 mM HEPES (pH 7.4) and 10 mM metal salt aqueous solutions (or 1 mM TACN amd metal salt solutions). Necessary amounts of 100 mM HEPES and water were added to the reaction mixture, and then 10 μl of plasmid water solution (0.5 μg DNA). Total reaction volume was 50 μl. Cleavage was performed at ambient temperature in the dark for 15 h. After the process was over, 10 μl of loading buffer (0.1% Bromophenol Blue in 50 % aqueous glycerol, 100 mM TBE) was added to the reaction mixture, and 25 μl aliquot of the resulting solutiuon was applied to 0.8 % agarose horizontal gel electrophoresis. Running buffer for electrophoresis was TBE (100 mM tris-borate, 2.5 mM EDTA) containing 0.5 μg/ml ethidium bromide). The gels were run at constant voltage (150 V) for 4 h. Gel slabs were washed with 200 ml of water for 30 min to remove excess dye. The nucleic acid bands were visualized under UV light by ethidium bromide fluorescence and quantitated via fluorescence imaging. The extent of DNA degradation was determined using GeneTools software. A correction factor of 1.47 was used to account for the decreased ability of ethidium bromide to intercalate into form I DNA versus forms II and III [21].

Synthesis of TACN-oligonucleotide conjugate ***5***. TACN carboxylic derivative **3** (0.25 μmol), BOP (1.75 μmol, 7 eq), HOBT (1.75 μmol, 7 eq) and N-methylmorpholine (3.75 μmol, 15 eq) were dissolved in 150 μl of dry DMF and incubated

for 45 min at 37 °. Then the solution of the activated ester was added to 6 A_{260} (50 nmol) of 5'-aminoalkyl-modified oligonucleotide T_{15} in 350 µl of 20 mM MOPS buffer (pH 7.5). The reaction mixture was incubated at 37 ° for 4 h with occasional stirring. Then 50 µl of 3M sodium acetate (pH 5) was added followed by 1.5 ml of methanol. The mixture was cooled (-20 °C, 20 min) and centrifuged. The precipitate was washed with methanol (2x500 µl) and dissolved in 200 µl of 0.5 M NaOAc (pH 5). The crude product was precipitated with 600 µl of ethanol, cooled (30 min at -20 °) and collected by centrifugation. The pellet was washed with EtOH (200 µl) and dissolved in 500 µl of conc. ammonia and incubated at ambient temperature overnight. The ammonia solution was applied to the PD-10 column (Sephadex G-25, Pharmacia) and oligonucleotide conjugate was eluted with 50 mM TEAB buffer (pH 7.5). Eluate was evaporated, then co-evaporated with ethanol (2x5 ml) to decompose excess TEAB. Product **5** was purified by standard 20 % polyacrylamide gel electrophoresis. The isolated yield was 2.8 A_{260} (47 %).

Reaction of TACN-oligonucleotide conjugate with poly-A. To 1.2 A_{260} of TACN-T_{15} conjugate **5** (0.01 µmol) in 200 µl of 50 mM HEPES (pH 7.4) was added 0.015 µmol of $CuSO_4$ or $EuCl_3$ (15 µl of 1 mM water solution). In 10 min, 15 A_{260} of poly-A in the same HEPES buffer (100 µl) was added. The reaction mixture was incubated at ambient temperature for 15 h. Control experiment was performed with no metal addition. 10 mM EDTA (10 µl) was added to the mixture to quench the reaction, and aliquots were analyzed by reverse phase HPLC on the column Hypersil BDS C18 (3 µm, 4.6x50 mm, ThermoHypersil, Bellefonte, PA) in the gradient 0-30 % CH_3CN in 50 mM triethylammonium acetate buffer (pH 7.5), flow rate 1 ml/min. HPLC was run at 35 °C to ensure an efficient separation of T_{15} from the complementary poly-A chains.

Acknowledgement. This project has been supported by JREI (grant GR/R05574 «Macrocyclic metal complexes as a part of anti-sense DNA probe»).

Надійшла в редакцію 24.01.2007 р.

Металокомплекси 1,4,7-триазациклононану та їх олігонуклеотидні кон'югати як хімічні нуклеази

І.Я. Дубей, Л.В. Дубей, О.В. Пілецька[1], С.А. Пілецький[1]

Інститут молекулярної біології і генетики НАН України
вул. Акад. Заболотного, 150, Київ, 03143, Україна

[1] Кренфілдський центр здоров'я, Кренфілдський університет
Сілсоу, Бедфордшир, MK45 4DT, Великобританія

Резюме. Вивчено ефективність розщеплення нуклеїнових кислот комплексами 1,4,7-триазациклононану (TACN) із рядом перехідних металів у фізіологічних умовах. Показано, що макроциклічні комплекси міді(II), європію(III) і тербію(III) активно розщеплювали дирибонуклеотид ApA, однак комплекси інших досліджених йонів металів (Co^{2+}, Fe^{3+}, Mn^{2+}, V^{3+} та Zn^{2+}) не виявляли нуклеолітичної дії. Комплекси TACN із усіма вивченими металами, за винятком цинку, демонстрували нуклеазну активність щодо дволанцюгової ДНК плазміди pBR322. Ефективне розщеплення ДНК показали комплекси лантанідів, які, ймовірно, діють швидше за гідролітичним, ніж за оксидативним механізмом. У той же час комплекси Red-Ox-активних металів (особливо заліза, міді й ванадію) можуть каталізувати окислювальне розщеплення ДНК. Комплекс ванадію виявився одним із найактивніших ДНКаз. Отримано модельний кон'югат TACN із дезоксиолігонуклеотидом T_{15} пост-синтетичною конденсацією N-карбоксиалкільної похідної біс(N-трифторацетил)-захищеного азамакроциклу з 5'-аміноалкіл-функціоналізованим олігонуклеотидом. Європієвий комплекс T_{15}-зв'язаного TACN продемонстрував здатність розщеплювати комплементарну поліаденілову кислоту.

Ключові слова: хімічні нуклеази, металокомплекси, триазациклононан, розщеплення нуклеїнових кислот, олігонуклеотидні кон'югати.

References

1. *Sigman D.S., Mazumder A., Perrin D.M.* Chemical nucleases // Chem. Rev. — 1993. — Vol. 93, No. 6. — P. 2295-2316.

2. *Pratviel G., Bernadou J., Meunier B.* Carbon-hydrogen bonds of DNA sugar units as targets for chemical nucleases and drugs // Angew. Chem. Int. Ed. Engl. — 1995. — Vol. 34, No. 7. — P. 746-769.

3. *Cowan J.A.* Chemical nucleases // Curr. Opin. Chem. Biol. — 2001. — Vol. 5, No. 6. — P. 634-642.

4. *Chen C.B., Milne L., Landgraf R., Perrin D.M., Sigman D.S.* Artificial nucleases // ChemBioChem. — 2001. — Vol. 2, No. 10. — P. 735-740.

5. *Сильников В.Н., Власов В.В.* Конструирование реагентов для направленного расщепления рибону-

клеиновых кислот // Успехи химии. — 2001. — Т. 70, № 6. — С. 562-580.

6. *Liu C., Wang M., Zhang T., Sun H.* DNA hydrolysis promoted by di- and multi-nuclear metal complexes // Coord. Chem. Rev. — 2004. — Vol. 248, No. 1-2. — P. 147-168.

7. *Morrow J.R., Iranzo O.* Synthetic metallonucleases for RNA cleavage // Curr. Opin. Chem. Biol. — 2004. — Vol. 8, No. 2. — P. 192-200.

8. *Nittymäki T., Lönnberg H.* Artificial ribonucleases // Org. Biomol. Chem. — 2006. — Vol. 4, No. 1. — P. 15-25.

9. *Chin K.O.A., Morrow J.R.* RNA cleavage and phosphate diester transferification by encapsulated lanthanide ions: Traversing the lanthanide series with lanthanum (III), europium (III), and lutetium (III) complexes of 1,4,7,10-tetrakis(2-hydroxyalkyl)-1,4,7,10-tetraazacyclododecane // Inorg. Chem. — 1994. — Vol. 33, No. 22. — P. 5036-5041.

10. *Deal C., Burstyn J.N.* Mechanistic studies of dichloro(1,4,7-triazcyclononane) copper (II)-catalyzed phosphonate diester hydrolysis // Inorg. Chem. — 1996. — Vol. 35, No. 10. — P. 2792-2798.

11. *Blake D., Fallis I.A., Gould R.O., Parsons S., Ross S.A., Schroder M.* Selective derivatisation of aza macrocycles // Dalton Trans. — 1996. — No. 23. — P. 4379-4387.

12. *Hay E., Givan N.* The [Cu-([9]aneN$_3$)(OH$_2$)$_2$]$^{2+}$catalysed hydrolysis of the phosphotriester 2,4-dinitrophenyl diethyl phosphate // Polyhedron. — 1998. — Vol. 17, No. 4. — P. 463-468.

13. *McCue K.P., Voss D.A., Marks C., Morrow J.R.* Dinuclear copper (II) complexes that promote hydrolysis of GpppG, a model for the 5'-cap of mRNA // Dalton Trans. — 1998. — No. 18. — P. 2961-2963.

14. *Young M.J., Chin J.* Dinuclear copper(II) complex that hydrolyses RNA // J. Am. Chem. Soc. — 1995. — Vol. 117, No. 42. — P. 10577-10578.

15. *Qian J., Gu W., Liu H., Gao F., Feng L., Yan S., Liao D., Cheng P.* The first dinuclear copper(II) and zinc(II) complexes containing novel Bis-TACN: syntheses, structures, and DNA cleavage activities // Dalton Trans. — 2007. — No. 10. — P. 1060-1066.

16. *Selvi P.T., Stoeckli-Evans H., Palaniandavar M.* Synthesis, structure and DNA interaction of cobalt(III) bis-complexes of 1,3-bis(2-pyridylimino)isoindoline and 1,4,7-triazacyclononane // J. Inorg. Biochem. — 2005. — Vol. 99, No. 11. — P. 2110-2118.

17. *Rammo J., Hettich R., Roigk A., Schneider H.-J.* Catalysis of DNA cleavage by lanthanide complexes with nucleophilic or intercalating ligands and their kinetic characterization // Chem Commun. — 1996. — No. 1. — P. 105-107.

18. *Komiyana M., Takeda N., Shigekawa H.* Hydrolysis of DNA and RNA by lanthanide ions: mechanistic studies leading to new applications // Chem. Commun. — 1999. — No. 16. — P. 1443-1451.

19. *Franklin S.J.* Lanthanide-mediated DNA hydrolysis // Curr. Opin. Chem. Biol. — 2001. — Vol. 5, No. 2. — P. 201-208.

20. *Rawlings J., Cleland W., Hengge A.C.* Metal-catalyzed phosphodiester cleavage: secondary ^{18}O isotope effects as an indicator of mechanism // J. Am. Chem. Soc. — 2006. — Vol. 128, No. 51. — P. 17120-17125.

21. *Bernadou J., Pratviel G., Bennis F., Girardet M., Meunier B.* Potassium monopersulfate and a water-soluble manganese porphyrin complex, [Mn(TMPyP)](OAc)$_5$, as an efficient reagent for th oxidative cleavage of DNA // J. Am. Chem. Soc. — 1989. — Vol. 28, No. 18. — P. 7268-7275.

22. *Sakurai H., Tamura H., Okatani K.* Mechanism for a new antitumor vanadium complex: hydroxyl radical-dependent DNA cleavage by 1,10-phenanthroline-vanadyl complex in the presence of hydrogen peroxide // Biochem. Biophys. Res. Commun. — 1995. — Vol. 206, No. 1. — P. 133-137.

23. *Branum M.E., Que L.* Double-strand DNA hydrolysis by dilanthanide complexes // J. Biol. Inorg. Chem. — 1999. — Vol. 4, No. 5. — P. 593-600.

24. *Hegg E.L., Burstyn J.N.* Copper(II) macrocycles cleave single-stranded and double-stranded DNA under both aerobic and anaerobic conditions // Inorg. Chem. — 1996. — Vol. 35, No. 26. — P. 7474-7481.

25. *Hall G., Husken D., Haner R.* Towards artificial ribonucleases: the sequence-specific cleavage of RNA in a duplex // Nucleic Acids Res. — 1996. — Vol. 24, No. 18. — P. 3522-3526.

26. *Nittymäki T., Kaukinen U., Virta P., Nikkola S., Lönnberg H.* Preparation of azacrown-functionalized 2'-O-methyl oligoribonucleotides, potential artificial Rnases // Bioconjugate Chem. — 2004. — Vol. 15, No. 1. — P. 174-184.

27. *Nittymäki T., Lönnberg H.* Sequence-selective cleavage of oligoribonucleotides by 3d transition metal complexes of 1,5,9-triazacyclododecane-functionalized 2'-O-methyl oligoribonucleotides // Bioconjugate Chem. — 2004. — Vol. 15, No. 6. — P. 1275-1280.

28. *Hovinen J.* Synthesis of carbon-3-substituted 1,5,9-triazacyclododecanes, RNA cleavage agents suitable for oligonucleotide tethering // Bioconjugate Chem. — 1998. — Vol. 9, No. 1. — P. 132-136.

29. *Kovacs Z., Sherry A.D.* A general synthesis of mono- and disubstituted 1,4,7-triazacyclononanes // Tetrahedron Lett. — 1995. — Vol. 36, No. 51. — P. 9269-9272.

30. *Di Vaira M., Mani F., Stoppioni P.* A general and very straightforward route to the selective N-functionalization of 1,4,7-triazacyclononane with imidazole groups. Crystal structure determinations of nickel(II), copper(II) and zinc(II) complexes // Inorg. Chim. Acta. — 2000. — Vol. 303, No. 1. — P. 61-69.

31. *Weisman G.R., Vachon D.J., Johnson V.B., Grombek D.A.* Selective N-protection of medium-ring triamines // Chem. Commun. — 1987. — No. 12. — P. 886-887.

32. *McGowan P.C., Podesta T.J., Thornton-Pett M.* *N*-Monofunctionalized 1,4,7-triazacyclononane macrocycles as building blocks in inorganic crystal engineering // Inorg. Chem. — 2001. — Vol. 40, No. 7. — P. 1445-1453.

33. *Yang W., Giandomenico C.M., Sartori M., Moore D.A.* Facile N-1 protection of cyclam, cyclen and 1,4,7-triazacyclononane // Tetrahedron Lett. — 2003. — Vol. 44, No. 12. — P. 2481-2483.

34. *Dubey I., Pratviel G., Meunier B.* Modification of the thiourea linkage of fluorescein-oligonucleotide conjugate to a guanidinium motif during ammonia deprotection // Bioconjugate Chem. — 1998. — Vol. 9, No. 5. — P. 627-632.

35. *Dubey L.V., Dubey I.Y.* Onium salts as coupling reagents in the preparation of silica polymer supports for solid phase oligonucleotide synthesis // Ukr. Bioorg. Acta. — 2004. — Vol. 1, No. 1-2. — P. 23-28.

36. Handbook of Biochemistry and Molecular Biology (Ed. By Fasman G.). — CRC Press: Boca Raton, FL, 1975. — 3rd Ed. — Vol. 1. — P. 175.