SYNTHESIS AND ANTISENSE ACTIVITIES OF DNA OLIGOMERS WITH HYDROLYTICALLY ACTIVE SITES

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Approval of the graduate school of Natural and Applied Sciences.

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I certify that thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

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Synthetic and sequence-specific ribonucleases, which hydrolze RNAs selectively at the target sites, have been synthesized. Various lanthanide complexes have been shown to promote phosphodiester hydrolysis. Encapsulated lanthanide complexes like 1,4,7,10-tetrakis- (carbamoyl)-1,4,7,10-tetraazacyclododecane (TCMC) complexes are highly stable under physiological conditions. Distribution of the available coordination sites of the lanthanide between the ligand and the labile water molecules has proved to be critical in determining the activity and stability of the complex in water at neutral pH. The ultimate goal being an *in vivo* application as a sequence-selective RNA/DNA cleaving agent (artificial RNAse/DNase), where the kinetic stability is a distinct advantage. The final goal

is to conjugate such hydrolytically active molecules to DNA oligomers, the design must include a functional group that can be made reactive in standard conjugation protocols. To that end, a nitrophenyl-derivatized TCMC-La^{3+} complex was synthesized, which is as active as the unmodified TCMC-La^{3+} in the transesterification of an RNA model substrate. The most important attribute of this compound is the nitrophenyl group, which would allow further derivatization and conjugation to a DNA oligomer, thus creating a potential for the sequence selective hydrolysis of its target. Later, this La^{3+}-complex was appended to the 5'-end of an oligonucleotide and the hydrolytic activity of the conjugate was proved under physiological conditions.

Another lanthanide complex, 1,4,7-Tris (carbamoyl methyl)-1,4,7-triazacyclononane complex of Ce(IV) is both stable in aqueous solutions at neutral pH and very efficient in promoting the hydrolysis of a phosphodiester model compound and yeast $tRNA^{phe}$.

Knowing that oligoamines efficiently hydrolyzes RNAs, due to intramolecular acid-base cooperation, conjugate of tris (2-aminoethyl)amine (TREN) and a DNA oligomer was prepared. It has been shown that this conjugate hydrolyzes the HIVTat mRNA at the target site under physiological conditions.

Keywords: artificial enzymes, phosphodiester hydrolysis, RNA hydrolysis, Lanthanide complexes, lanthanum (III) complex, cerium (IV) complex.

ÖZ

HİDROLİTİK AKTİF BİRİMLER EKLENMİŞ DNA OLİGOMERLERİNİN SENTEZİ VE ANTİSENSE AKTİVİTELERİ

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Bu çalışmada, RNA'yı sekans spesifik olarak hidroliz edebilecek yapay ribonukleazlar sentezlenmiştir. Birçok lantanid kompleksinin fosfodiester hidrolizi yaptığı gösterilmiştir. 1,4,7,10-tetrakis- (carbamoyl)-1,4,7,10-tetraazacyclododecane (TCMC) kompleksleri gibi enkapsüle edilmiş lanthanide kompleksleri fizyolojik koşullar altında oldukça kararlıdırlar. Mevcut lantanid koordinasyon bölgelerinin ligand ve su molekülleri arasındaki, dağılımı, kompleksin nötral pH'daki suda kararlılığı ve aktivitesinin belirlenmesinde kritik rol oynadığını kanıtlanmıştır. *In vivo* uygulamalarda en son amaç olarak, RNA ya da DNA'yı diziye özgün olarak kesebilen yapay RNaz ya da DNaz'ların kinetik kararlılıkları önemli bir avantajdır. Böylesi hidrolitik bakımdan aktif moleküllerin DNA oligomerlerine bağlanması amaçlandığından, dizayn mutlaka standart konjügasyon

protokollerinde aktive edebilecek bir fonksiyonel yapı içermelidir. Bu amaçla nitrofenil grubu eklenmiş TCMC-La^{3+} kompleksi sentezledi ve bu kompleksin modifikasyona uğratılmamış TCMC-La^{3+} kompleksi kadar model RNA substratlarının transesterifikasyonunda aktif oldukları gösteridi. Bu bileşiğin en önemli özelliği, bir DNA oligomerine bağlanmasına izin vererek, hedef bölgede sekans seçici hidroliz için bir potansiyel yaratmasıdır.

Bir diğer lantanid kompleksi, 1,4,7-Tris (karbamoyil metil)-1,4,7-triazacyclononane hem nötral pH'daki sulu çözeltilerde hem de fosfodiester model bileşiğinin ve de maya $tRNA^{Phe}$'sının hidrolizinin yürütülmesinde çok etkilidir.

Molekül içi asit-baz işbirliğinden dolayı oligoaminlerin RNA'yı etkili olarak hidroliz ettiği bilindiğinden oligo DNA'ya konjüge edilmiş tris (2-aminoethyl)amine (TREN) molekülleri hazırlandı. Fizyolojik koşullar altında bu bileşimin HIVTat mRNA'sını etkili bir şekilde hidroliz ettiği gösterildi.

Anahtar kelimeler: yapay enzimler, fosfodiester hidrolizi, RNA hidrolizi, Lantanid kompleksi, lantan (III) kompleksi, seryum (IV) kompleksi.

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μg	Microgram
ATP	Adenosine triphosphate
Bp	Base pair
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
H	Hour
Hrs	Hours
M	Molar
MCS	Multiple Cloning Site
min (s)	Minute (s)
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
ng	nanogram
NPAC	1-(4-nitrophenyl carbomoyl)-tris(carbomoyl)-1,4,7,10-tetraazacyclododecane
ODN	Oligo deoxyribonucleotide
PNK	Polynucleotide kinase
RNA	Ribonucleic acid
SDS	Sodium Dodecylsulfate
Sec	second
TCMC	1,4,7,10-tetrakis-(carbomoyl)-1,4,7,10-tetraazacyclododecane
TREN	tris(2-aminoethyl)amine

CHAPTER I

INTRODUCTION

1.1. Introduction

Enzymatic catalysis is in part due to the critically located reactive functional groups in the active site. This results in substrate selectivity, reaction selectivity, and stereoselectivity. Specific substrate binding can be achieved by metal coordination, or hydrogen bonding, electrostatic and hydrophobic interactions in aqueous solution.

Nature has chosen the ester linkage for lipids, the amide linkage for proteins, and the phosphate diester linkage for nucleic acids. Among the three types of linkages, phosphate diester linkage is the most stable one. Perhaps not suprisingly, it is also the most difficult to hydrolyze. Phosphodiester bond is the one that nature has chosen to preserve the genetic material. Over the years, there has been considerable interest in developing catalyst that can hydrolyze phosphodiesters.

Acceleration of non-enzymic phosphodiester hydrolysis by many catalysts (both organic and inorganic Cech and Herschlag, 1990; Yoshinari *et al.,* 1991; Morrow *et al.,* 1992) has received considerable

attention in recent years due to its projected impact in a number of fields, including potential applications in molecular biology (Sullenger and Cech, 1993) and gene therapy (Dropulic *et al.,* 1993; Meunier, 1996). Cech and Herschlag showed that DNA bound to an RNA enzyme derived from the self-splicing intervening sequence of *Tetrahymena thermophilia* is hydrolyzed sequence-specifically with a half-life of about 69 min. at 50 °C (Cech and Herschlag, 1990). It has been shown that oligoamines are simple and efficient catalysts for RNA hydrolysis (Yoshinari *et al., 1991*). Lanthanides and complexes were found to cleave aryl phosphates and RNA by a hydrolytic pathway, probably acting at the same time as acid and base catalysts (Morrow *et al.,* 1992).

RNA is hydrolyzed *in vivo* by ribonucleases *via* a two step reaction involving transesterification by the 2'-hydroxyl group as a nucleophile, with concomitant cleavage of the RNA strand, followed by the hydrolysis of the resulting 2', 3'-cyclic phosphodiester. During the reaction, the 2'-OH is deprotonated, and the leaving 5'-alkoxy group is protonated. Both steps can be accomplished by one round of base catalysis, for example by imidazole (two histidine residues, His^{12} and His^{119}, in ribonuclease) or a metal hydroxide (M^{+}-OH), (e.g., alkaline phosphatase) as illustrated in Figure 1. Hydrolysis of RNA is much more rapid than DNA in both chemical and enzymatic processes due to the anchimeric assistance of the ribose 2'-hydroxyl group.

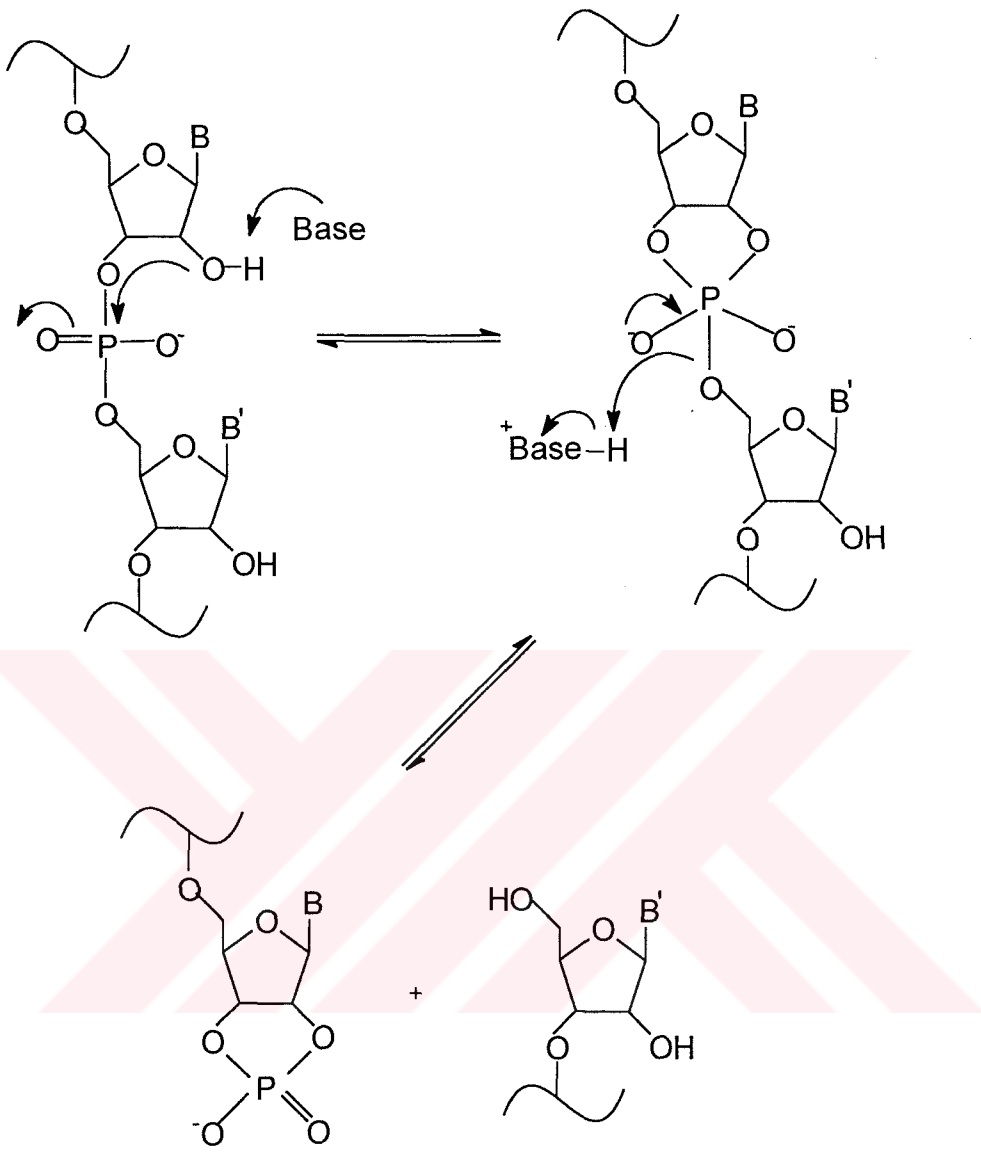

Figure 1: Transesterification of RNA.

1.2. Antisense Strategy in Artificial Ribonuclease Development

Gene expression in the cells is normally controlled by DNA-binding proteins, repressors, and activators. It is also found that the regulatory RNA sequences have been established as direct repressor of gene expression (Inouye, 1988).

Controlling the lifetimes of mRNA molecules is one of the means by which nature regulates protein synthesis (Belasco and Brawerman, 1993). The antisense method for controlling gene expression, which has great potential for use in antiviral chemotherapy, uses a similar idea: gene-specific deactivation or destruction of mRNA can inhibit the synthesis of harmful proteins (Cohen, 1989; Uhlmann and Peyman, 1990). Every gene gives rise to a relatively large number of copies of mRNA, which is translated into a large number of protein molecules. This is why inhibition of gene expression ought to be more efficient than inhibition of the resulting protein product.

Within the past two decades antisense oligonucleotides have emerged as a valid technology for sequence specific modulation of gene expression at the messenger RNA level, both *in vitro* and *in vivo* (Crooke, 1996; Zamecnik and Stephenson, 1978). Double helical DNA comprises two complementary oligonucleotide chains. Traditionally one of these chains, the sense strand, defines the genetic code, whilst the complementary chain, the antisense strand, provides the means of propagating that code, or in other words the template strand. The use of oligonucleotides in the treatment of certain viral diseases and types of cancer is based on inhibition of the unwanted protein translation by hybridization of the target mRNA molecule with a complementary oligonucleotide (Green *et al.,* 1986; Uhlman and

Peyman, 1990; Thuong and Helene, 1993; Lonnberg and Vuorio, 1996). When unmodified oligodeoxyribonucleotides are employed, the RNA/DNA duplex formed serve as a substrate for a cellular RNase H that cleaves the mRNA strand.

In natural form of this gene regulation method, an antisense RNA molecule is synthesized in the nucleus by transcription (Figure 2). Certain proteins are down regulated by the intracellular release of antisense RNA that is complementary to part of the target mRNA and prevents the message from being translated into protein at the ribosomes (Inouye, 1988). This gene regulation mechanism that relies upon the ability of messenger RNA, which is a single-stranded nucleic acid, is to be recognised in a gene specific manner by complementary nucleic acid molecules. This recognition occurs by the familiar and highly reliable Watson-Crick base pairing that is responsible for the specificity of the genetic code. Herein after, these developments have been improved further. Short, synthetically accessible oligonucleotides or analogs are designed to interact directly with mRNA, thereby selectively inhibiting the synthesis of the target protein. Naturally occurring antisense molecules do not meet the criteria for potential use in therapy and agriculture (De Mesmaeker *et al.*, 1995). If the nucleotide sequence of the target molecule is known, it is possible to write down directly the chemical formula of the inhibitor, corresponding to the base sequence of the antisense oligonucleotide.

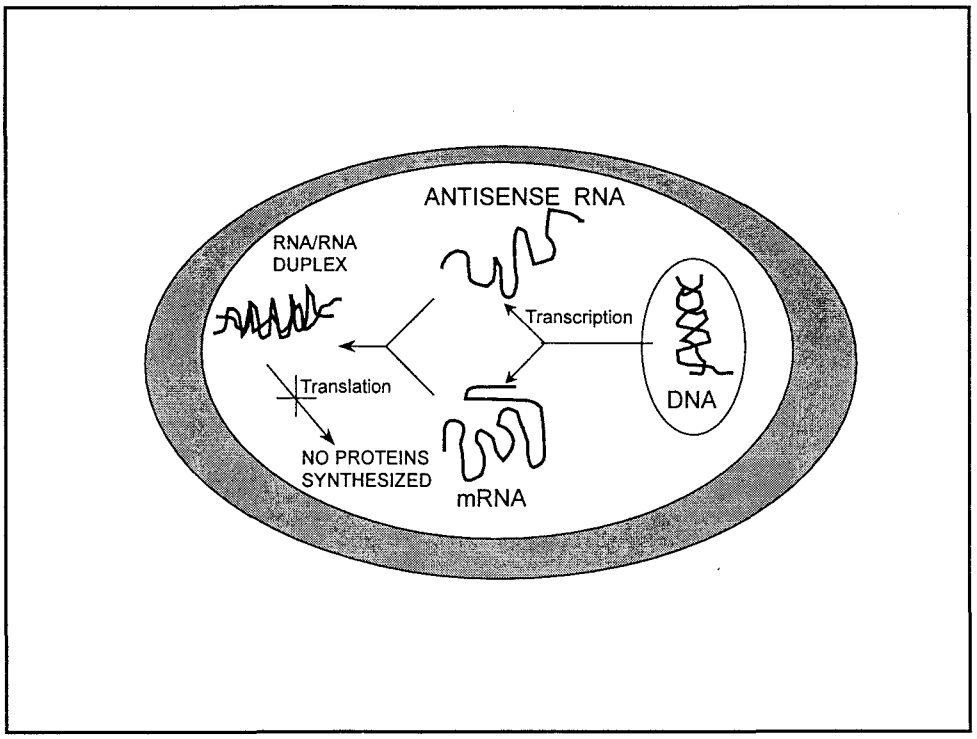

Figure 2: Synthesis of an antisense RNA molecule inside the cell.

The inhibitory effect of antisense oligonucleotide was first observed in 1978 by using a 13-mer oligonucleotide to inhibit the growth of Rous sarcoma virus in cell culture (Stephenson and Zamecnik, 1978). The chemical version (Figure 3) of the antisense technique employs antisense sequences that are synthesized outside the cell, using DNA synthesizers. For several reasons, including enhanced cellular uptake and lower cost, synthetic antisense molecules are much lower in molecular weight (typically 17-20 nucleotides long) than the full-length gene transcripts employed in the natural antisense method (Agrawal *et al* 1995).

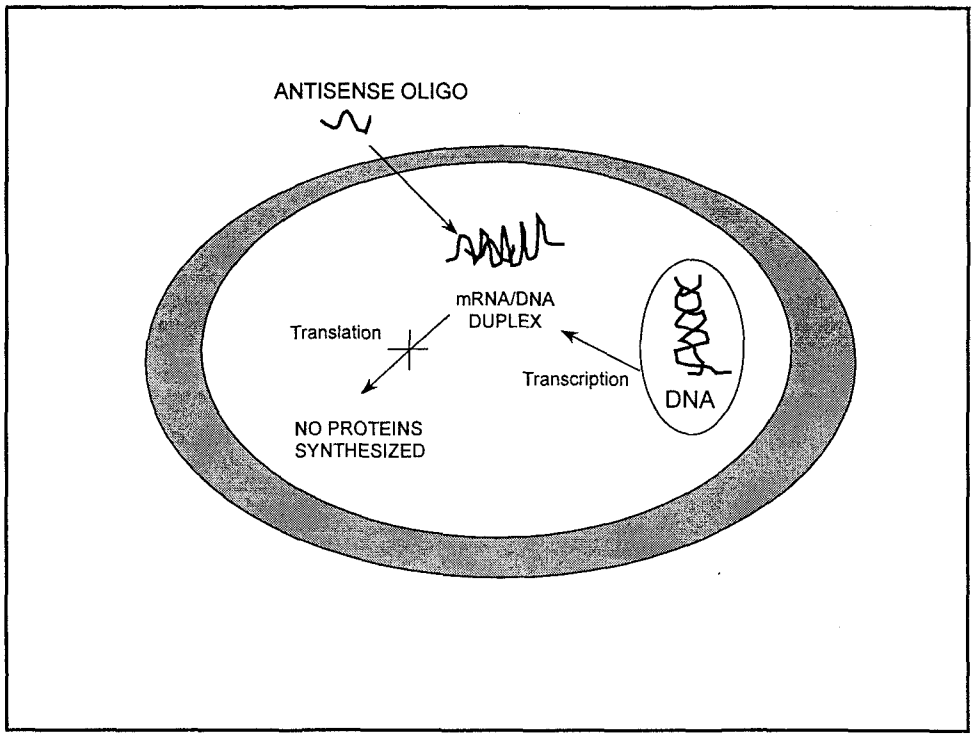

Figure3: The chemical version of the antisense technique.

As expected, artificial antisense RNA capable to inhibit the expression of any desired gene is widely used in fundamental research. However, it is also being investigated extensively with a view to use in therapy as well as in agriculture, especially in crop protection. The production of the "Flavr Savr tomato" is a good example of genetically altered food with the application of antisense method for the market. This genetically engineered tomato contains a gene that delays rotting. Tomato, like some other plant type produces the enzyme "polygalacturonase", an enzyme that digests pectin in the cell walls of tomato plants. This digestion induces rotting that is a part of the natural plant life cycle. Researchers at the firm Calgene, Inc., identified the polygalcturonase-encoding gene as a rotting agent,

removed the gene from plant cells and inserted it into a vector in opposite direction. Then a complementary copy of the gene is produced, isolated and inserted to tomato plant cells. Here it encodes an antisense mRNA molecule that hybridizes with and inactivates the sense mRNA molecule for polygalacturanase production. Because of the sense mRNA inactivation by the antisense molecule, there is no enzyme production for pectin digestion. As the pectin digestion is minimized, rotting slows down considerably. This use of antisense molecule was similar to that employed in drug research (Alcamo, 1996).

Interest in functional mimics of nucleases is driven by a variety of scientific and medical goals. Sequence-specific cleavage of RNA and DNA can be achieved by synthetic mimics: Direct degradation of the target RNA fragment can be achieved by oligonucleotides tethered to catalytically active groups. The oligonucleotide moiety recognizes a complementary base sequence in the target nucleic acid, and the catalytic group cleaves the chain over a narrow range of nucleotide units. Up to now, several such catalytically active oligonucleotide conjugates that hydrolyze the target RNA chain have been synthesized (Magda *et al.,* 1994; Bashkin *et al.,* 1994; Komiyama and Inokawa, 1994; Kazunari *et al.,* 1994; Hall *et al.,* 1994; Uchiyama *et al.,* 1994; Komiyama *et al.,* 1995; Hovinen *et al.,* 1995). By linking metal ion complexes covalently to oligodeoxyribonucleotides, it is possible to obtain an efficient site specific hydrolysis of the complementary RNA sequence, upon hybridization.

Development of new oligonucleotide chemistries has led to better analogs for antisense therapeutic applications (De Mesmaeker *et al.,* 1995). Since the pioneering work of Zamecnik and Stephenson (Zamecnik and Stephenson, 1978) on the use of antisense

oligonucleotides for the regulation of the gene expression, a large number of studies have been performed in this field targeting mRNA (antisense strategy) or DNA (antigene strategy) (Uhlmann and Peyman, 1990; Dervan, 1986). Many different problems have limited the development of the antisense therapy (oligonucleotide stability, sequence specificity, cellular uptake *etc.*). Antisense compounds must be stable and possess a reasonable half–life *in vivo*. DNA and RNA oligonucleotides are rapidly degraded by naturally occurring nucleases that hydrolytically cleave the phosphodiester backbone. In a pharmacological sense, mRNA is the receptor that can be targeted by oligonucleotides. Therefore a high binding affinity is crucial for such molecules. At the same time, the antisense oligonucleotide has ability to penetrate through the cell membrane. Once it reaches the cytoplasm it must bind specifically and with sufficient affinity to the target mRNA to inhibit its translation into the corresponding protein (Uhlmann and Peyman, 1990). Recent encouraging results have been obtained on animals, *e.g.* in the treatment of asthma with phosphorothioate antisense oligonucleotides (Nyce and Metzger, 1997; Rojanasakul, 1996).

1.3. Artificial Ribonucleases

A synthetic molecular construct that can cleave DNA or RNA sequence specifically is of great interest due to the potential applications in biotechnology and gene therapy (Sullenger and Cech, 1993; Dropulic *et al,* 1993). The development of such an "artificial enzyme" requires a hydrolytic unit which is capable of accelerating phosphodiester hydrolysis at nearly neutral pH around 37 °C and without a dependence on additives (oxidizing/reducing agents, high

concentrations of metal ions, *etc.*) of any kind which cannot be supplied *in vivo*. One remarkable feature of RNA transesterification and hydrolysis is that these reactions are catalyzed by a truly high range of species that span almost the entire periodic table. Protons, hydroxides, amines and other nitrogen derivatives, Mg(II), Ca(II), Fe(III), Ni(II), Cu(II), Zn(II), Pb(II), trivalent lanthanides, and UO_2^{2+} (Moss *et al.,* 1997)and Th(IV) salts (Ihara *et al.,* 1996) are just some of the species including enzymes and ribozymes known to cleave RNA through nucleophilic paths.

The design and synthesis of artificial agents, which possess nucleic acid polymers-cleaving activity, have also become an increasingly valuable approach for developing novel types of antitumor drugs (Sigman and Chen, 1990). The designed molecule is constituted of both a DNA oligomer and the moiety responsible for the sequence-selective cleavage of the target mRNA. The ribozyme mimics deliver their own catalytic function to the target RNA and do not depend on RNase H.

A number of different strategies have been employed by different groups worldwide; general acid/general base catalysis by simple amines (Yoshinari *et al.*, 1993) or guanidinium (Smith et al, 1993) derivatives, catalysis by lanthanide (Morrow *et al.*, 1992; Magda *et al.*, 1994; Chin and Morrow, 1994; Magda *et al.*, 1997) or Co^{3+} complexes (Chin and Zou, 1988; Chung *et al.*, 1990; Hettich and Schneider, 1997; Chin, *et al.*, 1989), all proved to be promising leads.

Functional mimics of ribonucleases are defined as synthetic molecules that cleave RNA in a sequence directed manner, using biomimetic chemical reactions such as transesterification and hydrolysis (Figure 4). For transesterification (Figure 4a), an alcohol or

alkoxide is the nucleophile, while for hydrolysis, water or hydroxide is almost always the nucleophile (Kimball *et al.*, 1993; Takasaki and Chin, 1994). Furthermore, most organic reactions that catalyze RNA transesterification go on to hydrolyze the resulting 2', 3'-cyclic monophosphate to a mixture of 2'-and 3'-phosphate monoesters, as indicated in Figure 4b.

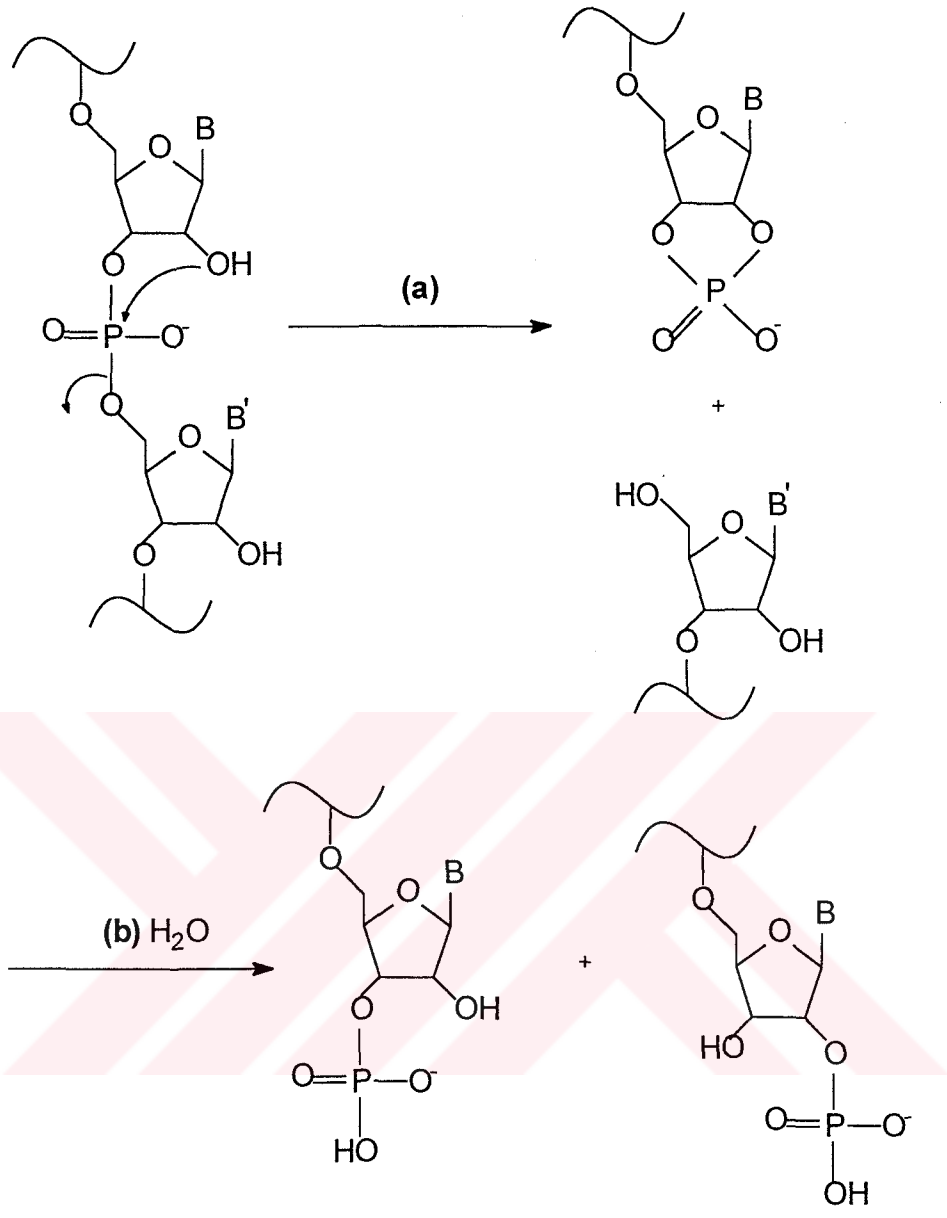

Figure 4: Nucleophilic cleavage of RNA: **(a)** Transesterification with concomitant cleavage of the phosphodiester backbone and **(b)** Hydrolysis of the 2',3'-cyclic monophosphate to a mixture of 2'-and 3'-monophosphates.

1.4. Sequence-Specific Cleavage by Artificial Ribonucleases

Selective scission of nucleic acids has been one of the most challenging topics, and many elegant artificial nucleases were reported (Povsic *at al.,* 1992 and references therein). In most of them oxidative cleavage of deoxyribose at the target site has been used, eventhough this kind of scission is unfavorable for application to molecular biology and biotechnology, for several reasons. First, the structures of the termini are different from those of the fragments obtained on enzymatic scission, and thus the resultant fragments cannot be ligated by use of ligase. Secondly, a clear-cut scission is rather hard to achieve since the active species (e.g., •OH) diffuse prior to the attack toward the ribose. Thirdly, the radical species tend to have harmful effects on biomaterials, when produced *in vivo*. Hydrolytic scission is desirable for versatile applications (Komiyama, 1995). Hydrolytic scission, exhibited by natural nucleases, has been mimicked rarely.

The potential for specificity is the greatest advantage of sequence-specific cleavage approach. Artificial ribonucleases have very important properties compared to their natural examples. One of the most important characteristics of such artificial enzymes is the high sequence-specificity, which can be much greater than those of natural restriction enzymes and ribonucleases. Thus, only a specific RNA can be selectively cleaved, all other RNAs remaining intact. Synthetic RNases for sequence-selective scission of RNA (Zuckermann *et al.*,1988 and Cech *et al.*,1986) can differentiate among various RNA molecules in cells from each other and cleave only one of them, and thus are applicable to selective regulation of the expression of a specific gene. Furthermore, artificial RNases, which recognize

oncogenes in cancer cells, should provide an effective and harmless therapy for cancer, since ideally they should have no effects on the RNAs of noncancerious cells.

Restriction enzymes recognize specific DNA sequences, usually 4-8 base pairs in length, and cut the DNA in a highly specific manner. However, the ability to specify only a 4-8 base sequence typically results in multiple cleavage sites in a genome, on plasmids and in other large DNA substrates. Ribozyme mimics allow cleavage specificity to be tuned simply by changing the length and content of the DNA sequence employed. Undoubtedly, these artificial enzymes should be powerful and essential tools for molecular biology and biotechnology in the future.

Sequence-specific RNA cleavage was developed in preliminary forms by at least two independent groups in the late 1980s (Cohen, 1989; Bashkin *et al.,* 1990). Stein and Cohen focused on imidazole-based approaches in their early-published suggestions and later experimental work (Cohen, 1989; Stein and Cohen, 1988), while Bashkin and co-workers developed a metal-based approach (Modak *et al.,* 1991). Other such as, Morrow, Chin and Komiyama pursued early work on nucleophilic cleavage of nucleic acids by well-defined metal complexes.

Concentration dependent non-specific interactions seem like an important problem on the further development of artificial ribonucleases. In some tissue culture experiments, it was shown that non-specific mechanisms dominate (Milligan *et al.*, 1993). Therefore, any reagent that can suppress the expression of a particular disease-causing gene at truly catalytic concentrations would be highly useful. An antisense oligonucleotide which is capable of catalytically

hydrolyzing the targeted RNA segment will be functional at lower concentrations and therefore will be less prone to the complications of non-specific interactions.

1.5. A Novel Oligo-Tetraamine Conjugate as Artificial Ribonuclease: Oligo-TREN

Simple amine catalysts yield appreciable cleavage (Komiyama *et al.*, 1989), but they often require elevated concentrations and/or temperatures (Breslow *et al.*,1986 and Yoshinari *et al.*, 1991). Likewise, polyamines such as polylysine and polyarginine peptides also yield significant cleavage (Barbier *et al.*,1992) but they are susceptible to cellular proteases.

It is known that the enzyme ribonuclease A cleaves RNA by the cooperative functioning of an immidazole base and an imidazolium ion acid, general acid general base catalysis.

It has been reported that (Yoshinari *et al.*, 1991) oligoamines efficiently hydrolysed RNAs, due to intramolecular acid-base cooperation. Two amino acid residues are required for the efficient RNA hydrolysis. It is strongly indicated that the present RNA hydrolysis involves an intramolecular attack by the 2'-hydroxyl group of the ribose. Thus, simple oligoamines are highly potent catalytic moieties for RNA hydrolysis as artificial ribonucleases. Furthermore, it was reported that oligoamines have been successfully used as the catalytic sites for sequence-specific artificial ribonucleases (Komiyama *et al.*, 1994). It has been proposed that those conjugates will

hydrolyze the substrate RNA exactly at target site under mild conditions (pH 8 and 37 °C).

As expected, TREN (tris (2-aminoethyl)amine) (Figure 5) is a unique tetraamine which has very unusual pK_a values as a result of through space and through bond electrostatic interaction. Only two methylenes separated the charges to be formed on protonation, and this result highly altered pK_a's. The values are 2.5, 4.7, 8.9 and 10.9. Therefore at pH's around neutrality, the TREN moiety carries two positively charged (protonated) and two neutral amino functions. Ammonium groups can act as general acids, and amine groups can act as general bases. Their cooperative effect may lead to a significant catalysis of hydrolysis. TREN molecule is effective for RNA hydrolysis due to such intramolecular cooperation. This residue is effectively concentrated at the target phosphodiester linkage due to a proximity effect, when the TREN is conjugated to a DNA oligomer and this conjugate together with the substrate RNA form a complex. Thus, concentration dependent problems can be overcome by this method.

The simplicity and the stability of the oligoamines are undoubtedly advantageous for the purpose of site specific cleavage (Komiyama *et al.*, 1997). The choice of TREN was based on our knowledge of the pK_a's of this amine, the values are such that at pH 7-8, there will be at least one protonated and one deprotonated amine ensuring a cooperative general acid/general base catalysis of RNA phosphodiester hydrolysis. There are studies showing that primary amine functions are efficient hydrolytic agents for RNA hydrolysis. In addition, it is also known that conjugation of DNA oligomers to amines (like poly-L-Lysine) improve their cell permeation characteristics (Curiel *et al.*, 1991). So, tetraamine derivatized oligos may also have improved permeability.

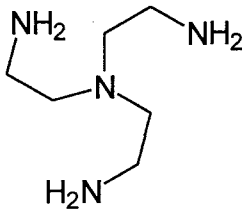

Figure 5: The structure of TREN (tris (2-aminoethyl)amine) molecule.

1.6. Lanthanide Ion Catalysis of Phosphodiester Hydrolysis

Lanthanide ions and their complexes have attracted attention as catalysts for phosphate hydrolysis because of the high efficiency of lanthanide ion catalysis. Especially La^{III}, Eu^{III} and Dy^{III} ions have remarkable catalytic activity, both complexed and as simple hydrated ions (Hayashi *et al.,* 1993; Morrow *et al.,* 1992; Magda *et al.,* 1992; Zhu *et al.,* 1988). The strong Lewis-acid lanthanides can neutralize the negative charge on the phosphate group, and at the same time supply a metal-bound hydroxide as a nucleophile. Of these catalysts, lanthanide (III) ions are characterised by enormously high catalytic activities (Komiyama *et al.,* 1992; Morrow *et al.,* 1992). There are numerous examples of metal-promoted hydrolysis of RNA. Several lanthanide ions and complexes promote transesterification/ hydrolysis of RNA dinucleotides, oligonucleotides, and RNA models; in some instances the reactions have been shown to be catalytic (Hegg, 1997; Morrow *et al*, 1992; Bashkin *et al.*, 1993 and Shelton *et al.*, 1991).

The positive charge of a metal ion is a key feature in the enzyme mimic that lowers the pK_a of coordinated water and provides a locally high concentration of the otherwise unavailable reagent OH^- at neutral pH (Figure 6). In RNA hydrolysis, the hydroxide ion bound to the lanthanide (III) ion (pK_a=8-9) (Burgess, 1978) functions as a general base catalyst, promoting nucleophilic attack of the 2'-OH residue of the ribose toward the phosphorous atom (Komiyama *et al.*,1992). In a similar manner, the positively charged metal centre can also serve as a general Lewis acid for activation of a substrate molecule, modulating its reactivity following coordination (a). Rate acceleration can occur by internal attack within the coordination sphere of the metal ion (super acid catalysis) (b) or by positioning of a substrate ligand near an essential group at the active site (c) (Lippard *et al.*, 1994). Furthermore, metal ions indirectly activate these reactions. A metal coordinated hydroxide can act as an intramolecular general base catalyst (d) or metal coordinated water molecules can act as an intramolecular general acid catalyst (e). Furthermore, the large positive charge of the lanthanide ion electrostatically stabilizes the negatively charged transition state of RNA hydrolysis; hydrolysis of phosphate esters is promoted by the adjacent positive charge, since the negative charge around the reaction center increases when the transition state is formed (Komiyama, 1995).

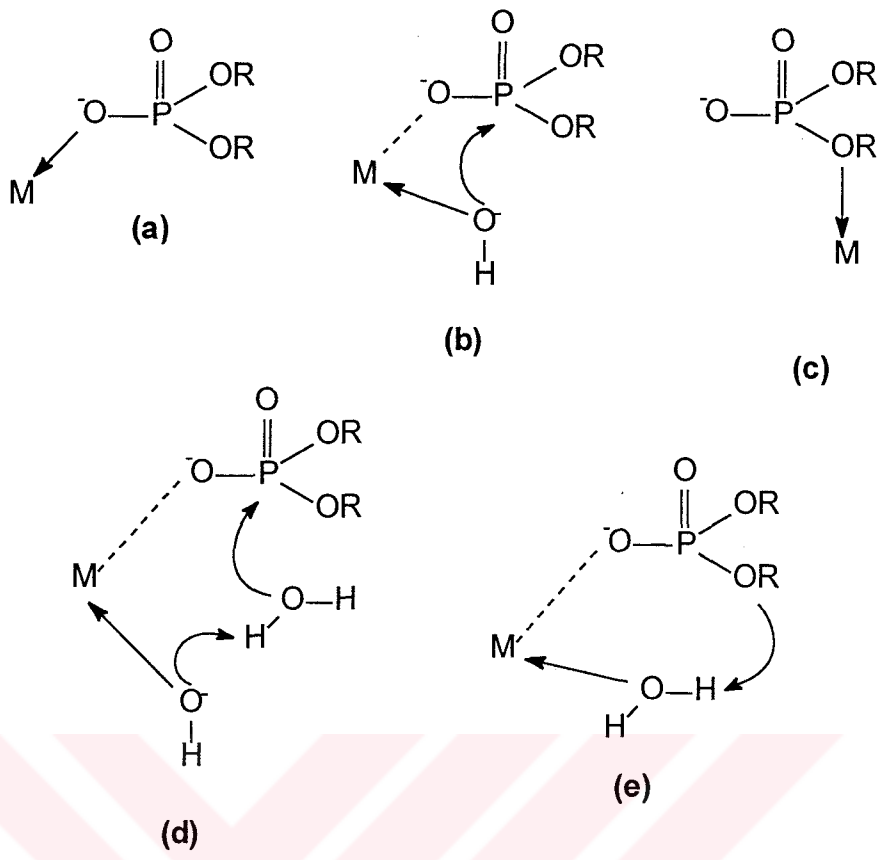

Figure 6: The roles of metal ions for hydrolyzing phosphodiesters where OR represents phenols or alcohols. **(a)** Single Lewis acid activation. **(b)** Metal hydroxide activation. **(c)** Leaving group activation. **(d)** Metal hydroxide acting as a general base catalyst. **(e)** Metal coordinated water acting as a general acid catalyst.

It is reasonable that lanthanide(III) ions should be most efficient metal centers in catalyzing and promoting the hydrolysis of phosphate esters, since lanthanide(III) ions have high ionic potential (Z^2/r), high coordination numbers and outstanding substitution lability (Scheneider *et al.*, 1993; Tasaki and Chin, 1994).

Several kinetic studies of phosphate diester transesterification by metal ions indicate that lanthanide ions are among the most efficient promoters (Morrow *et al.*, 1992; Komiyama *et al.,* 1992; Breslow and Huang, 1991). The trivalent lanthanides are good Lewis acids and have flexible coordination geometries and high cationic charge. The lanthanides are considered to be oxophilic metal ions and bind well to phosphate diesters. Oxophilicity is an important property for an artificial nuclease, as it is desirable that the metal ion binds to the phosphate ester in preference to binding a nitrogenous base of RNA. Thus, strong coordination of a metal complex to one or more nitrogenous bases of RNA may inhibit metal complex promoted transesterification. Because, this event renders the loss of the catalytic behaviour of lanthanide ions to facilitate phosphodiester transesterification by preventing the formation of lanthanide hydroxide complex at neutral pH. Lanthanide ions are good catalyst for the cleavage of the phosphodiesters which are normally resistant to nucleophilic reagents, presumably because the lanthanides supply both electrophilic acceptor sites for the phosphodiester's $P\text{-}O^-$, and metal ion bound hydroxyl (M-OH) nucleophiles for simultaneous attack at phosphorous and oxygen double bond (P=O) (Morrow *et al.*, 1992 and references therein).

Cerium ions and their complexes have been reported to hydrolyze dinucleotides, short oligonucleotides, and supercoiled plasmid DNA (Takasaki and Chin, 1994; Komiyama *et al.,* 1995). Reactions with cerium(III) seem to require oxygen whereas reactions with cerium(IV) do not, because cerium(III) is oxidized to cerium(IV) and the cerium(IV) is the active species. The quadruple positive charge of cerium(IV) stabilizes the negatively charged transition state for the phosphodiester hydrolysis. With larger charge/size ratio Ce(IV)

is expected to cause larger rate accelerations in the phosphodiester hydrolysis, but Ce(IV) salts at neutral pH, immediately form ceric-hydroxide gel, (Sumaoka, *et al.,* 1994) both slowing down the reaction and complicating the kinetic characterization of hydrolysis. The gel formation could be avoided by using nonionic Brij-35 micelles (Bracken *et al.,* 1997) or working at acidic pH values, both approaches considering the potential applications, would not offer optimal solutions.

1.7. Requirements for the Ligands to be Used in the Catalysis of Hydrolysis

The design of inorganic compounds that may be useful as therapeutic or diagnostic agents is a topic of great interest in bioorganic chemistry. The utility of macrocyclic ligands in controlling the reactivity of metal ions and in forming highly stable metal complexes make them ideal for use in the design of new metallodrugs. Strongly chelating ligands may serve to maintain the metal ion in solution in an active form. Because free metal ions may be highly toxic, many pharmaceutical applications require the use of strong chelates

Artificial metalloribonucleases require ligands, which effectively deliver the metal ion to the vicinity of the RNA strand. It has been reported that artificial metalloribonucleases whose metal complexes are linked to the oligonucleotides *via* a linker effectively induce RNA cleavage. La^{+3} makes strong coordination with the amide groups of tetraamide macrocycle, TCMC (1,4,7,10-tetrakis-(carbamoyl)-1,4,7,10-tetraazacyclododecane) (Amin, *et al.*, 1994) (Figure 7).

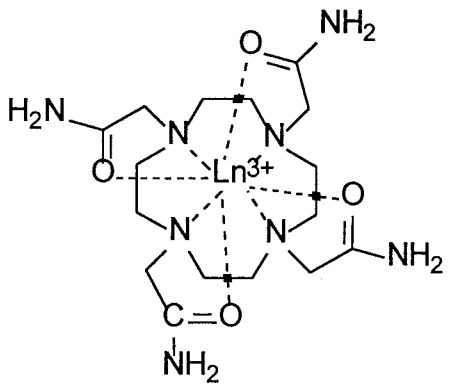

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Figure 7: The structure of TCMC (1,4,7,10-tetrakis- (carbamoyl)-1,4,7,10-tetraazacyclododecane). Ln = La, Eu, Dy

Ligands for the lanthanides must strongly chelate lanthanide ions but not inactivate them as catalysts. Coordination sites must be available for catalysis and the metal ion should retain a high degree of Lewis acidity. An overall positive charge on the complex may aid in catalysis. Although lanthanide ions themselves show no specificity, there is a significant stereospecificity when they form complexes with appropriate ligands.

Lanthanide macrocyclic complexes must be inert to metal ion release in water. The most well known class of lanthanide macrocyclic compounds are the crown ethers. Lanthanide complexes that will efficiently promote RNA cleavage will require available coordination sites for catalysis and must be inert to metal ion release or have large formation constants.

Macrocyclic ligands that form thermodynamically stable or kinetically inert complexes with metal ions are used in the design of metal complexes that behave as artificial ribonucleases. A motivation for the study of metal complexes that catalyze RNA cleavage lies in the development of sequence-specific cleaving agents for RNA.

The aqueous chemistry of the lanthanide (III) ions (Ln(III)) is dominated by their oxophilicity. In aqueous solution, saturated or unsaturated neutral nitrogen donors have a low affinity for lanthanides. However, when incorporated into a chelate ring containing an oxygen donor, the neutral nitrogen donor readily coordinates to lanthanides.

N-substituted octadentate ligands derived from 1,4,7,10-tetraazacyclododecane form kinetically robust square-antiprismatic complexes in aqueous solution with metal ions that prefer coordination number of eight. The functionalization of polyazamacrocycles with pendant chains containing donor atoms, which can coordinate, is a powerful technique to modify the chemical and physical properties of the metal ion (Bernhardt *et al.*, 1990). When the N substituents contain amide groups, the neutral ligand forms tripositive complexes capable of binding further to one water molecule. (Amin *et al.*, 1995 and Parker *et al.*, 1995). The ten-coordinate La^{3+} ion may attain a higher coordination number and thus has more coordination sites remaining for binding substrate and for catalysis. Such complexes have been considered as artificial nucleases. The ten-coordinate La^{3+} ion is bound to four nitrogen and four oxygen atoms from the TCMC ligand, an oxygen atom. The $[La(TCMC)]^{3+}$complex contained two available coordination sites and promoted rapid RNA cleavage. In addition, the design of La^{3+}-macrocycle includes an ester group for potential coupling of an RNA recognition moiety (Smith *et al.*, 1993).

1.8. Lanthanide Ion Complexes

Metal complexes that cleave RNA *via* a hydrolytic mechanism have advantages over those that utilize an oxidative mechanism. Hydrolytic cleavage agents do not require co-reactants and do not produce highly reactive metal oxenes or radical species. Furthermore, because the 2'-hydroxyl on the ribose ring makes RNA nearly 100 000-fold more reactive toward hydrolysis than DNA (Thompson *et al.*, 1995).

Lanthanide complexes must be robust if they are to be useful as catalyst. They require available coordination sites for catalysis and must be inert to metal ion release or have a large formation constant.

Lanthanide complexes are very important in terms of their hydrolytic activity. It has been shown that, certain lanthanide salts are good catalysts for phosphodiester hydrolysis. In order to direct a hydrolytically active lanthanide complex to the desired region of a target RNA molecule, the complex should carry hydrolytically active and kinetically stable functional group. Thus, the complex should allow conjugation to an RNA complementary oligonucleotide fragment. Morrow (Amin, *et al.,* 1994) has shown that La^{3+} ion complex of a TCMC derived ligand, p-nitrobenzyl-tris(carbomylmethyl)cyclen (NBAC) (**27**) (illustrated in part 3.2. Figure 27) is not stable in aqueous solutions at neutral pH, due to the absence of 8^{th} donor atom.

Metal complexes have been shown to promote RNA cleavage rapidly at 37°C at neutral pH (Stern *et al.*, 1990). The overall charge on the complex may be important in catalyst design. The lanthanide

complexes that are active transesterification catalyst have neutral ligand.

Several types of macrocyclic lanthanide complexes have been developed which are inert to dissociation of the metal ion and hydrolyze the phosphodiester backbone of RNA as free complexes in solution (Amin *et al.,* 1994) as well as covalently attached to antisense oligonucleotides (Magda *et al.,* 1997; Komiyama *et al.,* 1995)

1.9. DNA Cleavage by Metal Ions and Their Complexes

The extremely long half-life times of DNA approaching more than 100 million years for total hydrolysis (Chin *et al.,* 1989) makes the development of supramolecular catalysts a significant challenge. DNA cleaving molecules, particularly those with a simple structure and high efficiency, have considerable potential in chemistry, molecular biology, and medicine (Dervan, 1986; Pratviel *et al.*, 1995). Therefore, much attention has been directed towards the design and synthesis of novel DNA cleaving molecules.

The most efficient nonenzymatic method of cleaving DNA is by an oxidative procedure (Sigman, 1986). In contrast, enzymes cleave DNA by catalyzing the hydrolysis of the phosphodiester bond.

DNA-cleaving metal complexes are usually activated in the presence of a reductant such as dithiothreitol or ascorbic acid to generate the reactive oxygen species responsible for DNA cleavage (radical reaction to decompose the sugar moiety). Recently, the groups of Komiyama and Chin reported that lanthanide (III) ions

induce DNA cleavage by hydrolysis of the phosphodiester of DNA (Matsumoto and Komiyama, 1992; Takasaki and Chin, 1994). These reports strongly support that the DNA cleavage by lanthanide (III)-complex may proceed *via* a hydrolytic mechanism.

The sequence specific recognition of double-stranded DNA is an essential biological process performed by DNA-binding proteins and involved in the regulation of transcription, replication, recombination, and DNA repair. The design of synthetic molecules that bind sequence specifically to unique sites on human DNA, thereby to some extend mimicking the action of the natural proteins, may have major implications for the treatment of genetic, oncogenic, and viral diseases. It has already been reported that metal ions and their complexes hydrolze RNA (Morrow *et al.*, 1992; Breslow and Huang, 1991; Young and Chin 1995; Baskin *et al.,* 1994). The phosphodiester linkages in DNA are so stable that reports of their nonenzymatic hydrolysis have been rather scarce (Basile *et al.,* 1987; Schnaith *et al.,* 1994). It was shown that lanthanide ions are effective in promoting DNA hydrolysis (Komiyama *et al.*, 1995; Rammo *et al.*, 1996). Cerium was especially prominent; the active species was subsequently shown to be the Ce(IV) ion (Takasaki and Chin, 1994). The hydrolytic character of DNA scission by Ce(IV) has been firmly established for the scission of dinucleotides (Takasaki and Chin, 1994). In the scission of a longer fragment of DNA, however, the evidence for the hydrolytic nature is less abundant. Although the resultant fragments seem to migrate along with the hydrolytic products in polyacrylamide gel electrophoresis, (Komiyama *et al.,* 1995). The possibility that the Ce(IV) ion, a well-known oxidant, oxidatively cleaves the deoxyribose is not completely ruled out by these results. The DNA might be chemically damaged somehow during the scission.

1.10. Sequence Selective Nucleophilic RNA Cleavage

In the last several years considerable progress has been made in terms of using oligonucleotide-appended, metal-based Lewis acidic functional groups to effect the site-directed cleavage of RNA.

Recent interest has focused on the molecular design of artificial nucleases, in which catalytic residues are attached to sequence-specific moieties (Dervan, 1986; Barton, 1986 and Zuckermann *et al.*, 1988). The specificity of these reagents is consequently derived from the Watson-Crick hydrogen bonding of the DNA strand to its complementary RNA sequence, and it exhibits the full specificity of the genetic code. The chemoselectivity of the mimics arises from the relative ease of nucleophilic cleavage of RNA vs DNA (Westheimer, 1987). This, in turn, is derived from the facile intramoleculer nature of the nucleophilic attack that typically drives RNA cleavage (Figure 4). DNA lacks the 2'-OH functionality and has proved almost completely inert to hydrolysis or transesterification by small molecule catalyst.

Over the years, many interesting artificial enzymes that hydrolyze the phosphate diester bonds of RNA have been reported. They include nonmetallic compounds and lanthanide complexes. Sequence selective hydrolysis of RNA has recently been achieved by using various metal complexes covalently attached to deoxyoligoribonuclotides using metal ion complexes that facilitate RNA cleavage. Site-specific hydrolysis of RNA has been realized using complementary DNA as a targeting molecule. When appropriate metal-binding sites were attached to DNA oligonucleotides, the addition of the lanthanide ions led to the site-specific hydrolysis of

complementary RNA oligomers (Bashkin *et al.*, 1994 and Magda *et al.*, 1994).

1.11. Ribozyme Mimics Based on Metal Complexes

Recently metal complexes have been attached to oligonucleotides to form ribozyme mimics. In 1994, the first example of a wholly synthetic, functional mimic of a ribozyme was reported (Bashkin, *et al.*, 1994). This mimic consisted of a 17-mer DNA oligonucleotide with a covalently attached terpyridine (terpy) ligand at C-5 of an internal uracil residue (Figure. 8). This mimic was synthesised using a modified DNA building block. The target was a 159-mer RNA sequence derived from the *gag*-mRNA of HIV, and sequence specific cleavage was observed at physiological pH and 45 °C in the presence of $CuCl_2$ over a period of 72 hrs . The cleavage was located at two positions within the duplex region opposite the modified base. These results were very important because they proved the concept that ribozyme mimics can be constructed by covalently linking RNA transesterification catalyst to a DNA oligomer. Therefore, the complex catalytic region of a natural ribozyme was replaced with a small molecule catalyst and the substrate recognition site was replaced with a DNA oligomer.

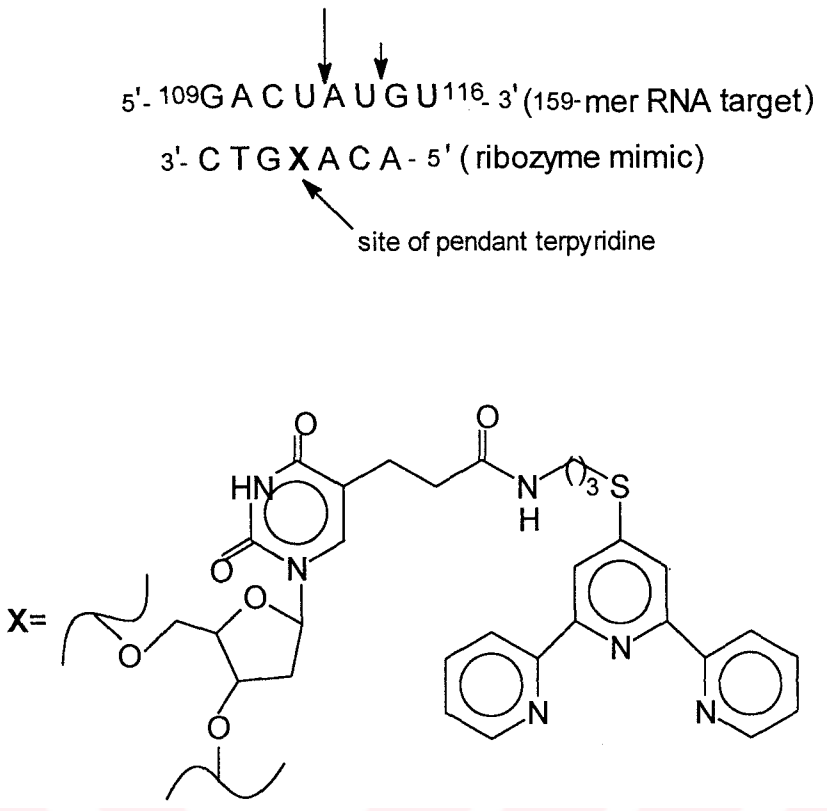
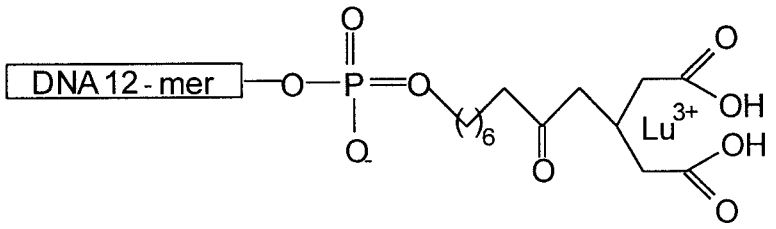


Figure 8: The first wholly synthetic, functional mimic of a ribozyme (Bashkin, *et al.*, 1994). The arrows represent Cu(terpy)OH^+ reaching across the major groove to cleave the target RNA.

Other examples of sequence-specific RNA cleavage agents based on metal complexes was reported by Matsumura *et al.* (Matsumura *et al.*, 1994). A 15-mer deoxynucleotide, which was functionalized at the 5' -end with a lanthanide-complexing iminodiacetate residue (DNA-IDA) (Figure 8), was prepared. This conjugate was synthesised using a postsynthetic strategy in which a DNA 15-mer with an amino group at its 5'-end was reacted with the 4-

nitrophenyl ester of the metal-complexing moiety. In the presence of various lanthanide ions (i.e., Lu(III), Th(III), and Eu(III)), the modified oligo cleaved a synthetic 39-mer RNA target outside the duplex region, opposite the metal complex. Reactions were performed at 37°C and pH 8, over a period of 8 hrs.



DNA – IDA

Figure 9: A DNA 15-mer functionalized at the 5'-end with an iminodiacetate residue.

Magda *et al.* (Magda *et al.*, 1994) synthesized ribozyme mimics by attaching Eu(III), chelated by a monoanionic, pentadentate texaphyrin ligand (EuTx), to 20-mer DNA oligos. Their postsynthetic modification strategy involved the synthesis of DNA oligonucleotides containing alkylamine groups either at C-5 of an internal thymine residue or at a 5'-terminal phosphate. This was followed by treatment of the deoxynucleotides with the europium(III)-texaphyrin carboxylic acid to affect amide coupling to the alkylamine groups (Figure 9). The conjugate with the texaphyrin complex attached at the 5'-end of the DNA strand site specifically cleaved a chemically synthesised 30-mer

RNA target near the expected location. Cleavage was observed at 37°C and pH 7.5 after 24 hrs. In contrast no cleavage was observed with the internally modified deoxyoligonucleotides. A major contribution from this work was the use of the stably preformed Eu(III) complex. Whereas other approaches required the addition of free metal ion cofactors for the cleavage event to take place, this method allowed the cleavage reaction to occur independently of such cofactors. This is extremely important when considering the use of ribozyme mimics for *in vivo* applications in the presence of competing protein ligands and other bioavailable metals.

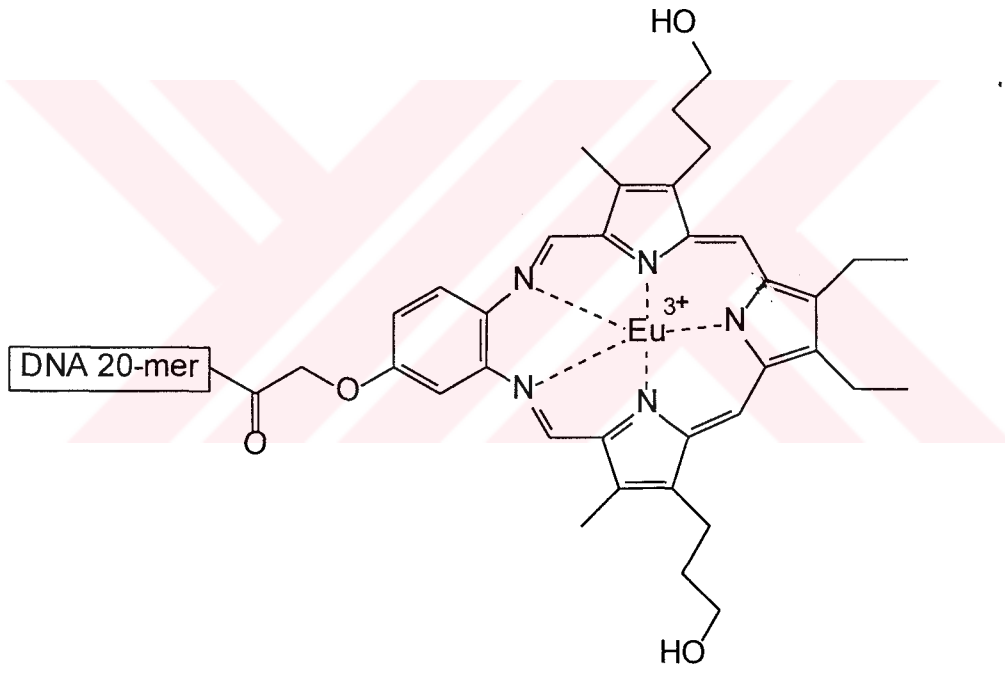


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Figure 10: Eu(III) texaphyrin conjugate used for sequence-specific RNA cleavage.

1.12. Assays for RNA cleavage

The substrates employed range from simple RNA model compounds to more complex ribopolymers, and the specific physical properties that are measured vary widely with the actual substrate under investigation.

1.12.1. Simple Functional Models of RNA for Hydrolysis studies

Comparative mechanistic studies of RNA with model phosphate ester compounds have been common (Morrow 1996, Chin 1989). This is partly due to the intrinsic simplicity and convenience of many models relative to true RNA substrates, which have a multiplicity of metal ion binding sites and many possible sites for hydrolytic cleavage. The compounds are designed to mimic the dialkyl phosphate esters that form the anionic backbone of RNA (Davies, *et al.*, 1988; Brown and Usher 1969; Breslow, *et al.,* 1989). The most frequently used models is illustrated in Figure 11. Those models contain good leaving groups upon transesterification, release aryloxides that allows studying by UV-vis spectrophotometry.

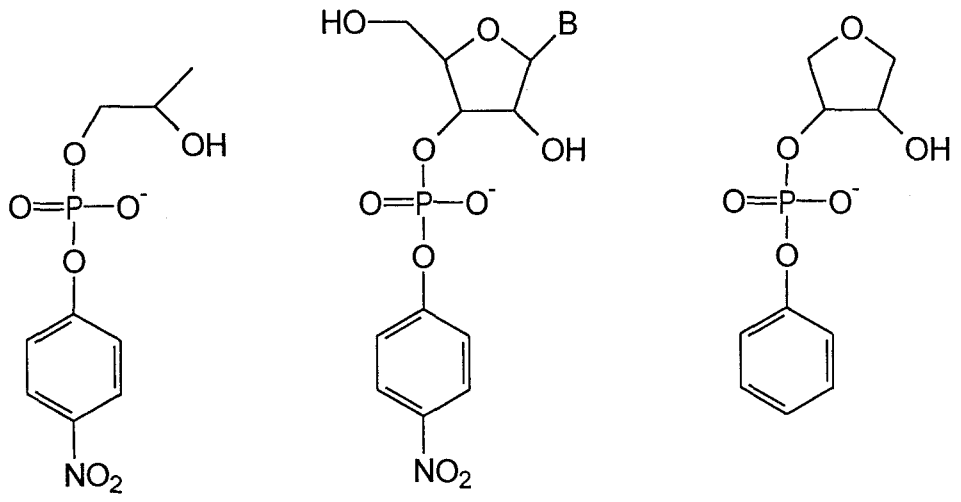

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Figure 11: RNA models for hydrolysis studies.

In addition to ease of analysis, the principal advantage of using RNA analogues with good leaving groups is that they usually exhibit enhanced cleavage rates. By comparison at neutral pH, transesterification of true RNA substrates is relatively slow, which makes it difficult to follow the progress of the reaction.

1.12.2.HIVTat Sequence as an Artificial Ribonuclease Target

Human immunodeficiency virus type-1 (HIV-1), like other lentiviruses, encodes a trans-activating regulatory protein, called Tat (transcriptional transactivator protein), that is needed for efficient transcription of the viral genome (Vaishnov *et al.*, 1991). HIV-Tat acts

by binding to an RNA stem-loop structure, the trans-activation response element (TAR), found at the 5' ends of all nascent HIV-1 transcripts. When bound to TAR, Tat alters the properties of the transcription complex, enhancing transcriptional initiation and processivity so that production of full-length viral RNA markedly increases. Tat functions primarily at the level of transcription elongation (Kao *et al.*, 1987; Laspia *et al.*, 1989; Marciniak *et al.*, 1991 and Cullen, 1990). Indispensability of Tat for HIV-1 replication makes it an attractive for antiviral drug development.

Consequently, the replication of human immunodeficiency virus type 1 (HIV-1) is dependent on the function of the viral transcriptional transactivator protein. Thus, blocking this highly important gene by a small molecule such as TREN and/or lanthanium complex is an attractive strategy for the inhibition of HIV.

1.13. Scope of the Study

In RNA targets like HIV genome, hydrolytic cleavage of the RNA could only be advantageous. We sought to develop an artificial enzyme that could be made to cleave almost any RNA substrate, efficiently and specifically under physiological conditions. Such a molecule could be used to inactivate a target RNA, probe a structural RNA, or assists in manipulation of recombinant RNA.

This study is directed towards the synthesis and characterization of such antisense agents. As target RNA, we chose a region of HIV-Tat (transcriptional *trans*-activator protein) a gene that encodes one of the most critical regulatory proteins of HIV and yeast $tRNA^{Phe}$. Tat is

essential for viral gene expression and replication and is therefore an attractive target for potential anti-viral agents.

This work differs from other studies in the field by several important respects. Our efforts are directed primarily towards the development of the hydrolytic oligo-amine conjugates and to the ligands that form sufficiently stable complexes and enhance the catalytic activity of the lanthanide La^{3+} complexes unlike the other complexes (Amin, *et al.,* 1994; Amin, *et al., 1995;* Amin, *et al.,* 1996). More effective and simple design of artificial ribonucleases were proposed. Besides they would function at physiological conditions. Also, DNA-appended hydrolytic groups will be powerful artificial ribonucleases in the cell.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

All reagents for chemical syntheses were purchased from commercial suppliers (Aldrich GmBH, Germany, unless otherwise noted) and used without further purification. All throughout this study, double distilled and deionized water that has the resistance of 18.2 Ω was used.

2.2. General Techniques

In this study, the novel compounds synthesized were characterized by Nuclear Magnetic Resonance (^{1}H, ^{13}C) Spectrum, Mass Spectrum, and Elemental Analysis.

^{1}H and ^{13}C-Nuclear Magnetic Resonance spectra were obtained using Bruker Gmbh DPX-400, 400 MHz High Performance Digital FT-NMR Spectrophotometer (TÜBİTAK Instrumental Analysis Laboratory) by using $CDCl_3$ or DMSO-d_6 as the solvent and TMS as an internal reference. Spin multiplicities are indicated by the following symbols: **s** (singlet), **t** (triplet), **m** (multiplet).

Mass spectra were acquired using with Fisons Instruments, VG Platform II LC-MS. (TÜBİTAK Instrumental Analysis Laboratory).

Elemental Analysis was performed using a Leco 932 CHNS-O Elemental Analysis Instrument (TÜBİTAK Instrumental Analysis Laboratory). Absorbance values were measured in Shimadzu UV-1601PC Spectrophotometer.

For the purification of the synthesized compounds, column chromatography was performed using Merck Silica Gel 60 (particle size: 0.040-0.063 mm, 230-400 mesh ASTM).

For monitoring of all the reactions, the Thin Layer Chromatography (TLC) technique was performed using Merck Silica Gel 60 F_{254} TLC Aluminum Sheets (20X20 cm).

2.3. General Procedures for Kinetics of Hydrolysis

The hydrolysis kinetics was studied by measuring the absorbance of p-nitrophenolate ion at 400nm. The absorbance spectra were recorded using a Shimadzu UV 1601 connected to a data station. In each experiment 4 mL of the buffered solution of the complex was placed in an optical cell, and the initial absorbance value was measured. This was followed by the addition of a stock solution of 2-hydroxypropyl-p-nitrophenylphosphate (HPNP) (40μL, 5mM). Absorbance data were collected for at least 3 half lives. Pseudo-first order rate constants (k_{obs}) were obtained by a computer program.

2.4. Syntheses

2.4.1. Synthesis of p-Nitrophenyl 2-hydroxypropyl phosphate

The RNA model compound, 2-hydroxypropyl-p-nitrophenylphosphate (HPNP) (**8**) that was prepared according to a literature procedure (Brown and Usher, 1965) was shown in Figure 12. A solution of disodium *p*-nitrophenyl phosphate (**6**) (1.32 gr, 5 mmol) in water (10 mL) was passed through a column (dimensions of the column: 23X3) of Dowex 50 WX8 (p.a; H^+-form; 200-400 mesh) (Fluka Biochemika) resin. Resin was suspended in 250 mL ddH_2O. In order to obtain clean resin, it was washed with 1 M HCl for five times in a sintered glass funnel. At the end of the washing steps, pH of the effluent was 5.0-7.0. For each case, 200 mL of 1 M HCl was used. The acid effluent from the column was brought to pH 8.0 with aqueous ammonia. 1,2-Epoxypropane (**7**) (20 mL) was added, and the solution was kept at 35 °C for 40 hrs. Unreacted epoxide was removed *in vacuo* and the mixture was passed down a column of Dowex 50 WX8 resin. The solution was neutralized (pH no higher than 7) with barium hydroxide solution (carbonate-free) and concentrated to about 10 mL *in vacuo* at room temperature. Ethanol (20 mL.) was added, and the precipitate of unchanged *p*-nitrophenyl phosphate was filtered off. The filtrate was concentrated to a small volume and added, with vigorous stirring, to a 10% ethanol in acetone (300 mL). The white precipitate of *p*-nitrophenyl phosphate was filtered off. The precipitation gave the pure product (**8**) of 580 mg, with 40% yield.

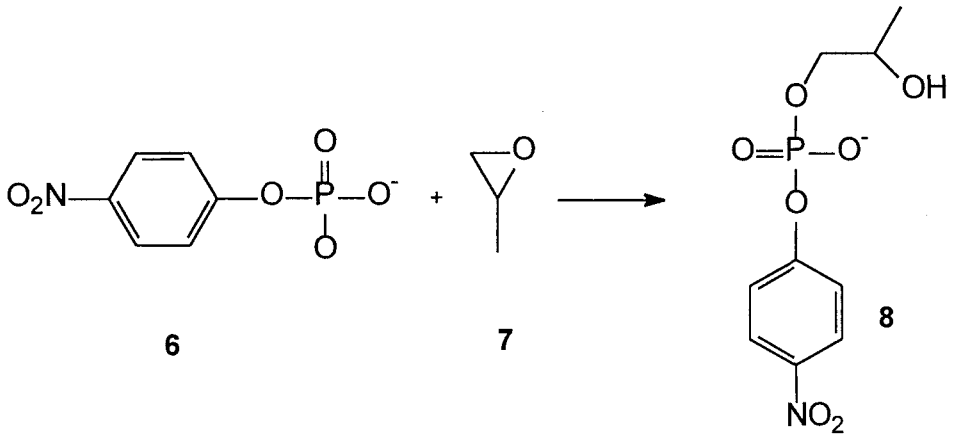


Figure 12: Synthesis of *p*-Nitrophenyl 2-Hydroxypropyl Phosphate (**8**).

2.4.2. Synthesis of the Polyamine Ligand 1,4,7,10-tetraazacyclodo-decanetetrahydrochloride

N,N', Tritosyl diethylenetriamine di sodium salt (**9**) (24.05 g) and N,N-Bis[2-(tosyloxy)ethyl]-toluene-4-sulfonamide (**10**) (20.84 g) was dissolved in DMF (140 mL). The resulting solution was heated at 120 °C for 2 hrs. After the solution was cooled down to the room temperature, cold water (1 L) was added and stirred vigorously. The precipitate was collected by filtration. All the precipitated tetratosylate (**11**) was dissolved in concentrated H_2SO_4 (325 mL). The solution was heated at 120 °C for 50 hrs while being stirred continuously. After H_2SO_4 treatment for the removal of tetratosyl group, the solution was cooled to room temperature, and then made alkaline by adding 8 M NaOH solution (1.86 L). The alkaline solution was left at room temperature for 12 hrs to complete the precipitation of Na_2SO_4. The precipitate was removed by filtration. The filtrate was divided into three. Each part was extracted with $CHCl_3$ (5X50 mL). Combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure, the residue was dissolved in ethanol (35 mL).

Concentrated HCl (8 mL) was added. The precipitate was collected by filtration, and washed with ethanol (10 mL). Shiny white flakes of the hydrochloride salt (**12**) (2.54 g) were obtained (Akkaya, *et al.*, 1989). The synthesis scheme is shown in Figure 13.

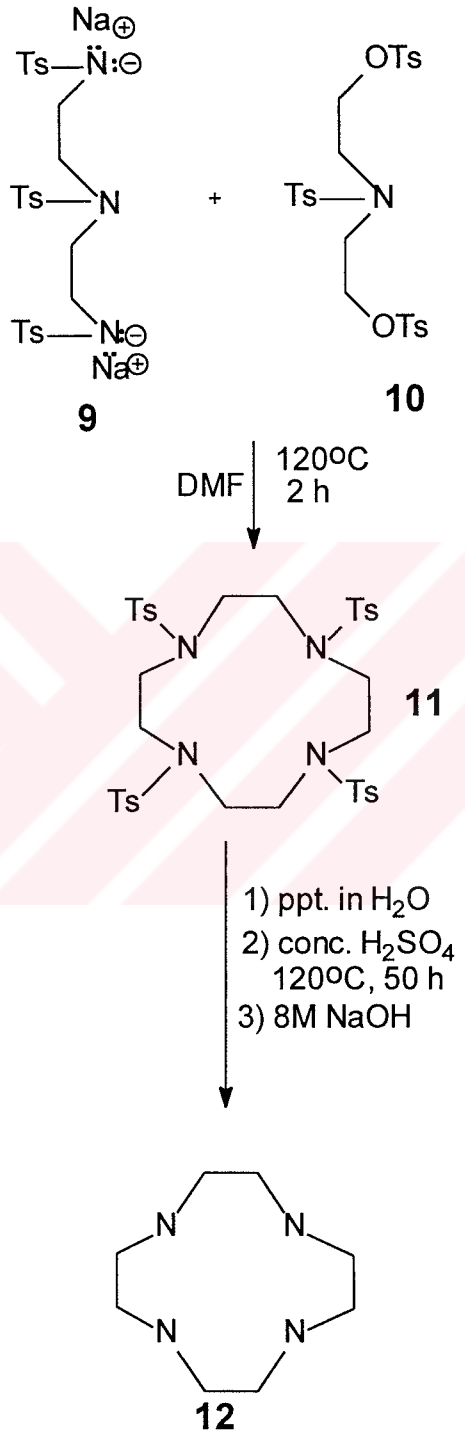


Figure 13: Synthesis of cyclen (1,4,7,10-tetra-azacyclododecane).

2.4.3. Synthesis of *p*-Nitrochloroacetanilide

p-Nitroaniline (**13**) (2.76 g, 20 mmol) and chloroacetic anhydride (**14**) (3.44 g, 20 mmol) were dissolved $CHCl_3$ (in 40 mL). Triethylamine (1 mL) was added, and the mixture was stirred at RT for 1 hour. The product (**15**) was collected by filtration. The schematic illustration of the synthesis is shown in Figure 14. The product, p-nitrochloroacetanilide was of satisfactory purity for use in the next step.

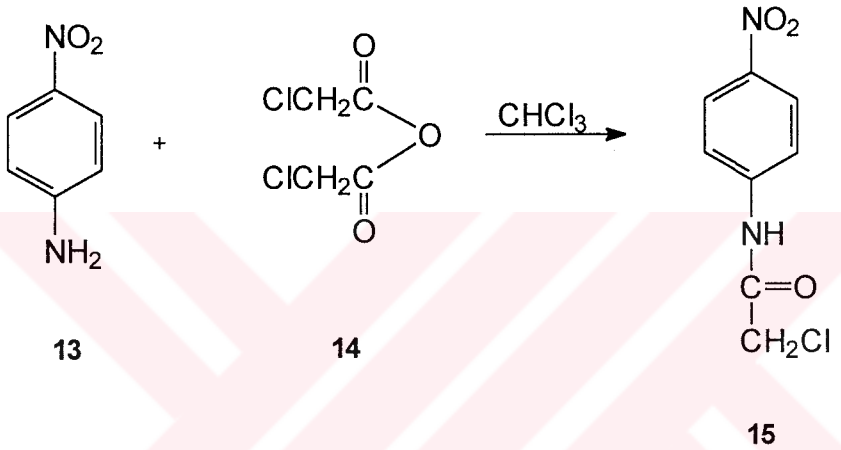


Figure 14: Synthesis of p-nitrochloroacetanilide (**15**).

2.4.4. Synthesis of 1-(4-nitro phenyl carbomoyl)-1,4,7,10-tetraaza cyclododecane.

A portion of the p-nitrochloroacetanilide (**15**) (1.5 g, 7.16 mmol) obtained was reacted with cyclen (1,4,7,10-tetraazacyclododecane) (**12**) (1.85 g, 10.75 mmol), in $CHCl_3$ (27 mL) at RT. The solvent was then removed under reduced pressure. The residue was applied to a slica-gel

column and the desired product (**16**) (Figure 15) was obtained by using $CHCl_3$/MeOH/conc. NH_3 as the mobile phase and collecting the appropriate fractions. The yield was 2.54 g (55%). ^{1}H-NMR (DMSO-d_6, 400.1 MHz) δ 2.52-2.70 (m, 16H, -CH_2CH_2-), 3.31 (s, 2H, (-CH2CO-), 7.99 (d, 2H, ArH), 8.23 (d, 2H, ArH) 10.5 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 100MHz) δ 46.2, 47.5, 47.8, 53.8, 59.6, 119.9, 125.7, 143.0, 146.0, 172.4. EI Mass Spectrum *m/e* 351 (M^++1).

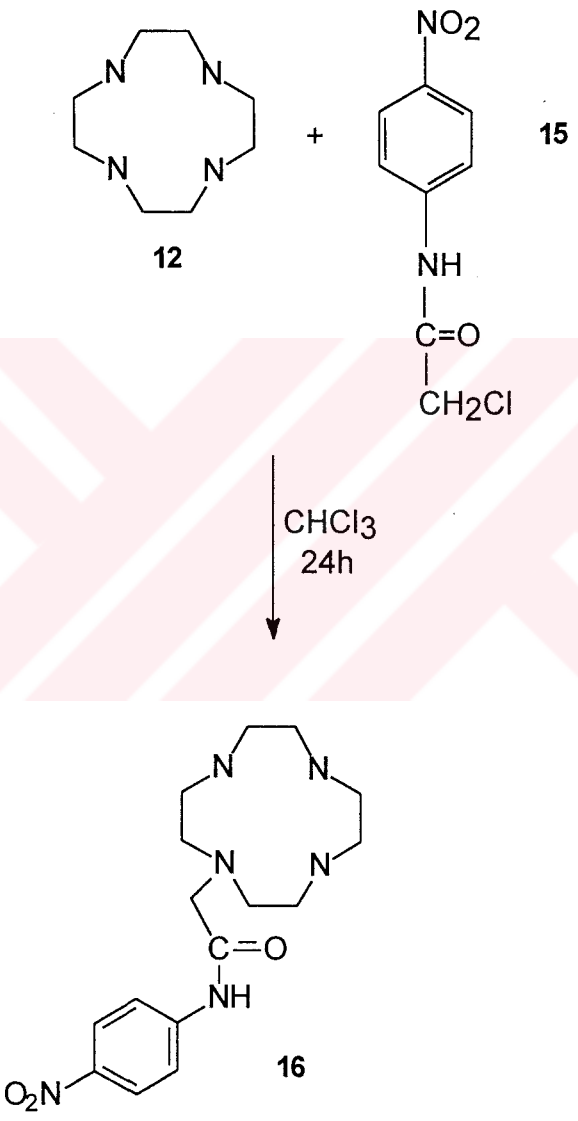


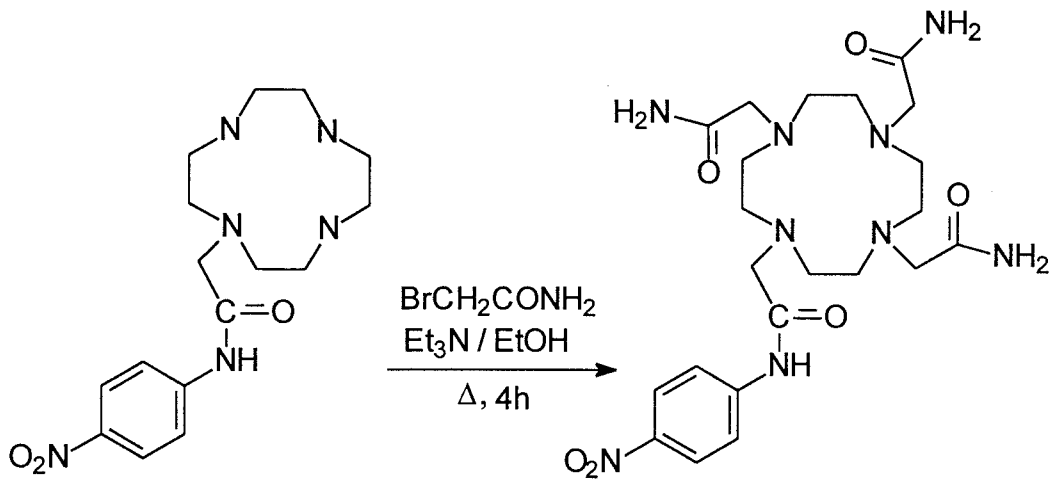
Figure 15: Schematic representation of synthesis of 1-(4-nitrophenyl-carbomoyl)-1,4,7,10-tetraazacyclododecane.

2.4.5. Conversion of 1-(4-nitrophenylcarbomoyl)-1,4,7,10-tetraazacyclododecane: Sulfate Salt to a Free Base

Carbonate (Na_2CO_3) buffer solution (1M, 30 mL, pH 10.5) was prepared. One quarter of the ligand obtained was dissolved in this buffer solution and extracted with $CHCl_3$ (4X30 mL). Then, the extract was dried over Na_2CO_3 and the solvent was removed under reduced pressure. This process was repeated three times more. Amount of the free base was 0.94g.

2.4.6. Synthesis of 1-(4-nitrophenylcarbomoyl)-tris(carbomoyl)-1,4,7,10-tetraazacyclododecane (NPAC)

1-(4-nitrophenylcarbamoyl)-1,4,7,10-tetraazacyclododecane (**16**) (2.52 mmol, 0.88 g) was suspended in anhydrous ethanol (40mL). To the mixture, bromoacetamide (8.55 mmol, 1.18 g) and 1.5 mL diisopropylethylamine was added. The reaction mixture was then heated under reflux for 4 hrs. On cooling to RT, the ligand (**17**) precipitated out of the solution. Further purification was achieved by recrystallizing the material from hot ethanol. Yield was 1.1 g (82%). The schematic illustration of the synthesis reaction is given in Figure 16. ^{1}H-NMR (DMSO-d_6, 400.1 MHz) δ 2.60-2.76 (m, 16H, -CH_2CH_2-), 2.90-3.05 (m, 6H, (-CH_2CO-), 3.25-3.40 (br s, 2H), 6.70 (br s, 3H, NH), 7.49 (m, 3H, NH), 7.91 (d, 2H, ArH), 8.22 (d, 2H, ArH), 10.48 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ 53.8, 58.5, 58.8, 59.5, 119.9, 125.8, 143.1, 145.7, 171.1, 173.6.



16 17

Figure 16: Schematic illustration of the synthesis of 1-(4-nitrophenyl carbomoyl)-tris(carbomoyl)- 1,4,7,10-tetraaza cyclododecane (NPAC) (**17**).

2.4.7. Synthesis of NPAC-La(III) complex

A solution of $(CF_3SO_3)_3La$ (lanthanum triflate, 0.123 g, 0.21 mmol) was prepared and added to a stirred suspension of the ligand NPAC (**17**) (0.11 g, 0.21 mmol) in ethanol (75 mL). To complete the complex formation, the reaction mixture was heated at reflux for 4 hrs after the dissolution of the ligand NPAC (**17**). The complex (**18**) was obtained in the form of a light yellow powder after the removal of the solvent under the reduced pressure and trituration with DCM and hexane (Figure 17). The yield was 0.2 g (90%). ^{1}H-NMR (DMSO-d_6, 400.1 MHz) δ 2.1-3.8 (br m, 24, -CH_2CH_2-, and –CH_2CO-), 7.92 (d, 2H, ArH), 8.22 (m, 3H, NH), 8.35 (d, 2H, ArH), 8.50 (s, 2H, NH), 8.58 (s, 1H, NH), 10.50 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ 56.9, 62.1, 121.7, 126.3, 143.7, 144.6, 174.7, 177.7.

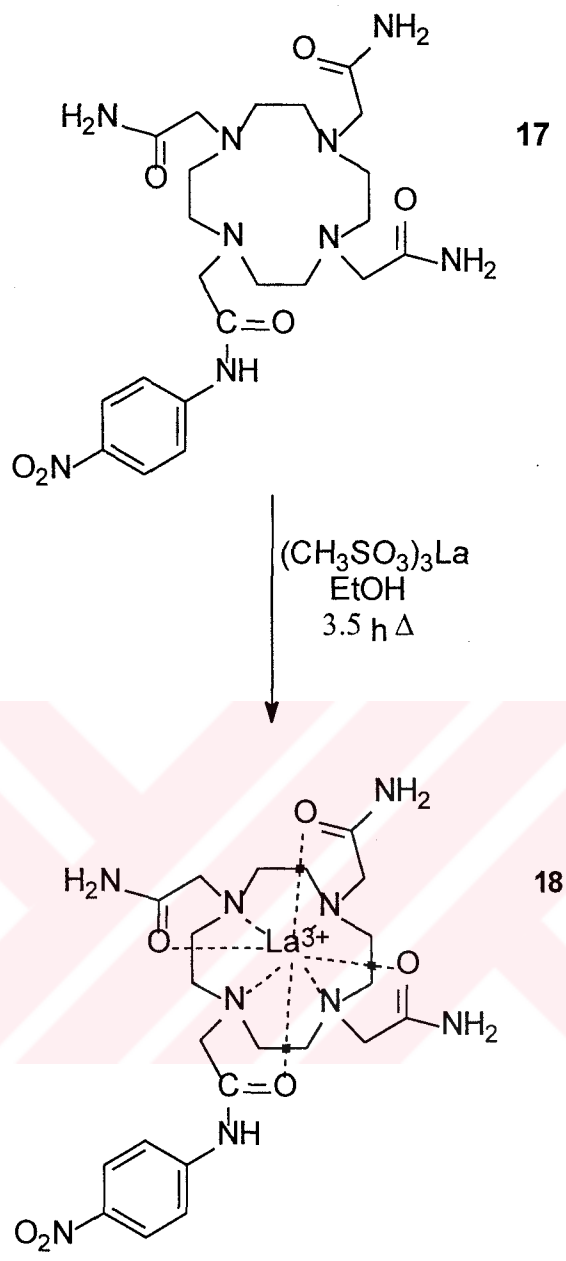


Figure 17: Schematic representation of the synthesis of 1-(4-nitrophenyl-carbomoyl)-tris(carbomoyl)-1,4,7,10-tetraaza-cyclododecane (NPAC)-La^{3+}-complex (**18**).

2.4.8. Reduction of NO_2 group of the ligand NPAC to NH_2 for coupling of the 16-*mer* DNA

The La^{3+}-complex (**18**) (0.8 g) was dissolved in ethanol (30 mL). To the complex solution of 10% Pd-charcoal (0.8 g) and cyclohexene (6 mL) were added. Then the solution was refluxed for 4 hrs. After the reflux, the solution was filtered and the solvent was removed under reduced pressure (Figure 18). ^{1}H-NMR (DMSO-d_6, 400.1 MHz) δ 2.0-3.6 (br m, 24, -CH_2CH_2-, and –CH_2CO-), 6.12 (br s, 2h, ArNH_2), 6.91 (d, 2H, ArH), 7.12 (m, 3H, NH), 7.37 (d, 2H, ArH), 8.45 (s, 2H, NH), 8.48 (s, 1H, NH), 10.33 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ 55.7, 61.6, 115.6, 122.3, 137.6, 141.5, 173.8, 176.2.

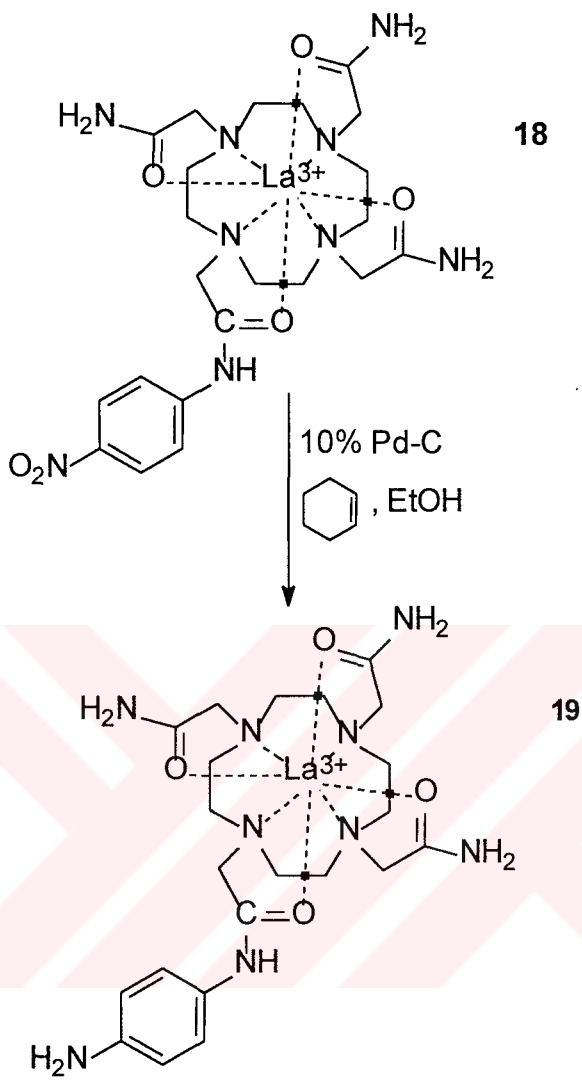


Figure 18: Reduction scheme of NO_2 group of the ligand NPAC to NH_2 for attachment of the 16-*mer* DNA.

2.4.9. Conjugation Reaction of 1-(4-nitrophenyl cabomoyl)-tris (carbomoyl)-1,4,7,10-tetraazacyclododecane (APAC)-La^{3+}Complex to a HIV-Tat Complementary 16-*mer* Oligodexynucleotide

The 5'-end amino modified oligo DNA (16-*mer*) (1 mg) was dissolved in Tris·Cl buffer (2 mL, 50 mM, pH 8.5). The structure of the amino modifier on the oligo was shown in Figure 19. The need for this amino modifier is to make the conjugation of the oligo DNA with the La3+-complex (**20**) possible.

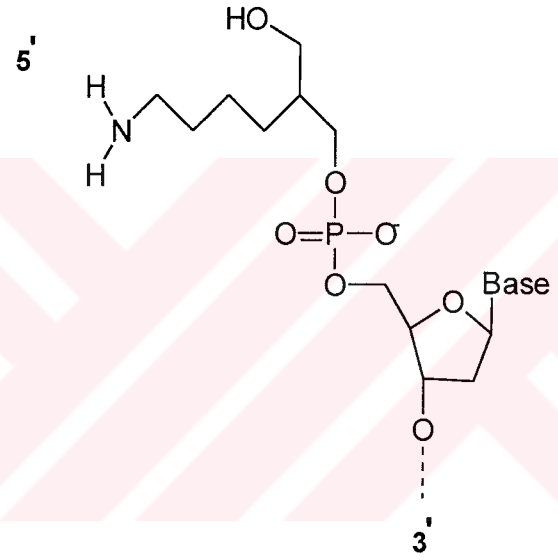


Figure 19: The structure of the amino modifier on the 16-*mer* oligo DNA.

The APAC La^{3+} complex (**19**) (0.073mmol) was dissolved in H_2O (1.94 mL). The, pH of the solution was adjusted to 7-8 using pH strips by the help of N,N-diisopropylethylamine (DIEA) (Sigma). After pH adjustment, to this solution, 90μL of bromoacetylbromide in $CHCl_3$ (1.94 mL) was added by stirring the complex solution. The pH of aqueous phase was adjusted to 7

with additions of DIEA and it was stirred vigorously for 5-10 min. At the end of this process, a layer formation was observed and was separated. The aqueous phase was extracted with $CHCl_3$. The pH of the aqueous layer was adjusted 2 with HCl (3 M) and then, extracted 5 times with equal volumes of ether. The pH was readjusted to 2 with HCl (3 M) and the aqueous phase was extracted five times with ether (this process was continued until the pH remained constant.). The residual ether was removed under the vacuum. The pH of the solution was adjusted to 5 with DIEA. Later, the solution (containing complex, **20**) was divided into aliquot and stored at -70 °C.

Half of the above complex solution was added into 2 mL of oligo solution (preparation of the solution was described above) for the conjugation reaction. The pH was adjusted to 8.5-9.0 with DIEA by using pH strips and incubated at 37 °C for 2hrs for the formation of the chimeric structure made up of an 16-*mer* oligo DNA and the La^{3+}-complex (**20**).

Half of the total 3 mL of the conjugation reaction mixture (oligo+ NPAC-La^{3+} complex) was employed to recover the oligo-conjugated complex *via* ethanol precipitation and dissolved in ddH_2O (1.5 mL).

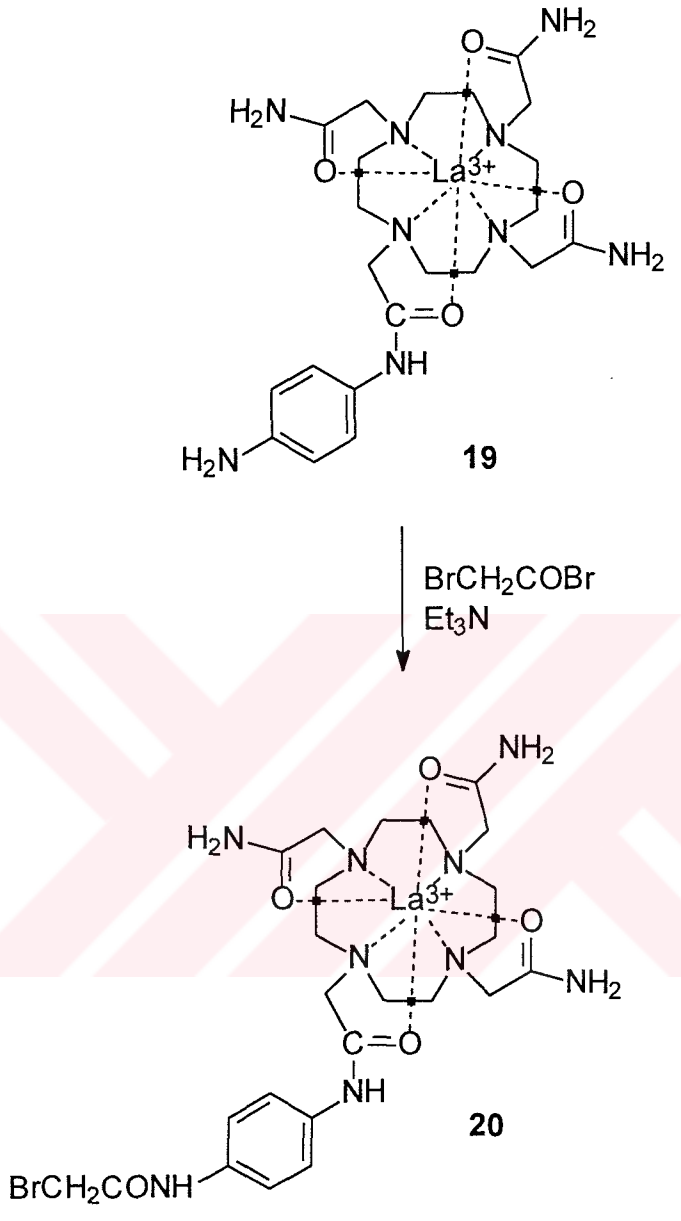


Figure 20: The reaction of the NPAC-La^{3+} complex with bromo-acetyl-bromide: a key step for coupling reaction with 16-*mer* DNA

SYNTHETIC 30-*mer* RNA

5'...TCC TAC GT**A AGG AAG AAG CGG AGA** CAG CGA...3'

Figure 21: Sequence of the target 30-*mer* RNA. The ribozyme mimic, oligo-lanthanum complexes comlementary to the boldface region

2.10. Conjugation Reaction of TREN (tris(2-aminoethyl)amine) Molecule to a 12-*mer* oligo DNA.

An artificial RNase (chimera of TREN and a DNA oligomer) was prepared in situ from a tris(aminoethyl)amine attached DNA oligomer. 12-*mer* oligo DNA is complementary with a portion of the substrate 30-*mer* synthetic RNA molecule, which has the same sequence with a part of the HIV-Tat mRNA (Figure 22).

5'	ATG	GAG	CCA	GTA	GAT	CCT	AGA	CTA
GAG	CCC	TGG	AAG	CAT	CCA	GGA	AGT	CAG
CCT	AAA	ACT	GCT	TGT	ACC	AAT	TGC	TAT
TGT	AAA	AAG	TGT	TGC	TTT	CAT	TGC	CAG
CTG	TGT	TTC	ATA	ACA	AAA	GCC	TTA	GGC
ATC	TCC	TAC	**GTA**	**AGG**	**AAG**	**AAG**	CGG	AGA
CAG	CGA	CGA	AGA	GCT	CAT	CAG	AAC	AGT
CAG	ACT	CAT	CAA	GCT	TCT	CTA	TCA	AAG
CAG	TAA	3'						

Figure 22: Sequence of the target HIV-Tat mRNA.. The TREN-oligo DNA conjugate is complementary to the boldface region.

TREN attached DNA oligomer was prepared by reacting 1-methyl imidazole (0.1 M) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (0.1 M) with 10 μL of 1114.7 pmol/μL of 5'-phoshorylated 12-mer DNA at 20 °C for 24 hrs. After the reaction TREN attached oligo DNA was ethanol precipitated and washed with 200 μL of 95% cold ethanol. Then, the oligo-TREN (Figure 23) was dissolved in 100 μL of DEPC treated ddH_2O.

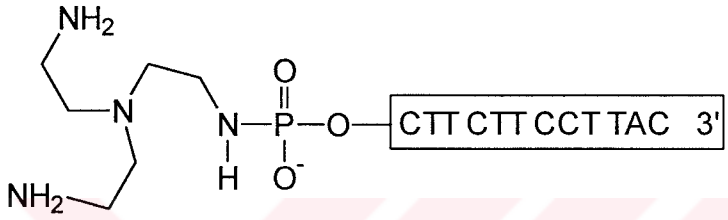


Figure 23: Oligo-TREN conjugate.

2.4.11. Synthesis of the Cerium Complex (1,4,7-Tris(carbamoylmethyl)-1,4,7-triazacyclononane-Ce^{IV}): TCMT

1,4,7-Triazacyclononane (**21**) (0.150 g, 1.16 mmol) was dissolved in ethanol (12 mL) and to this was added triethylamine (4 mL) and bromoacetamide (0.542 g, 3.93 mmol). The reaction mixture was refluxed for 6 hrs and during the course of the reaction a white precipitate formed (Amin *et al.*,1996). The product (**22**) was dissolved in boiling solution of 80% ethanol and 20% water (50 mL). The solution volume was reduced to 20 ml.

Crystals of the macrocycle were obtained and dried under vacuum. Yield 63%. ^{1}H NMR (D_2O) 2.71 (s, 12H, NCH_2 ring), 3.24 (s, 6H, NCH_2 amide pendent group). ^{13}C (D_2O) 50.43 (NCH_2 ring), 57.87 (NCH_2 amide pendent group), 174.51 ($CH_2C(O)$).

Ce(IV) complex (Figure 24) was prepared by simply refluxing a solution of the trialkylated azacrown ligand (TCMT) (0.09 g, 0.3 mmol) with $Ce(NH_4)_2(NO_3)_6$ (0.164 g, 0.3 mmol) in ethanol (120 mL) for 3.5 hrs. During the course of the reaction, complete dissolution of the TCMT ligand and $Ce(NH_4)_2(NO_3)_6$. Removal of the solvent under reduced pressure, followed by trituration with hexane (10 mL) resulted in Ce(IV) complex in the form of a light yellow powder. Satisfactory analytical data were obtained for the nitrate salt of the complex. ^{1}H NMR (CD_3CN): 3.01-3.21 (m, 12H, NCH_2 ring), 3.98 (s, 6H, NH_2 amide pendent group). ^{13}C (CD_3CN): 57.2 (NCH_2 ring), 65.4 (NCH_2 amide pendent group), 185.2 ($CH_2C(O)$).

The hydrolytic activity of the complex was studied using the phosphodiester model compound 2-hydroxypropyl-p-nitrophenylphosphate (HPNPP). Deionized, deaerated water was used in all buffers to minimize the possible oxidative cleavage.

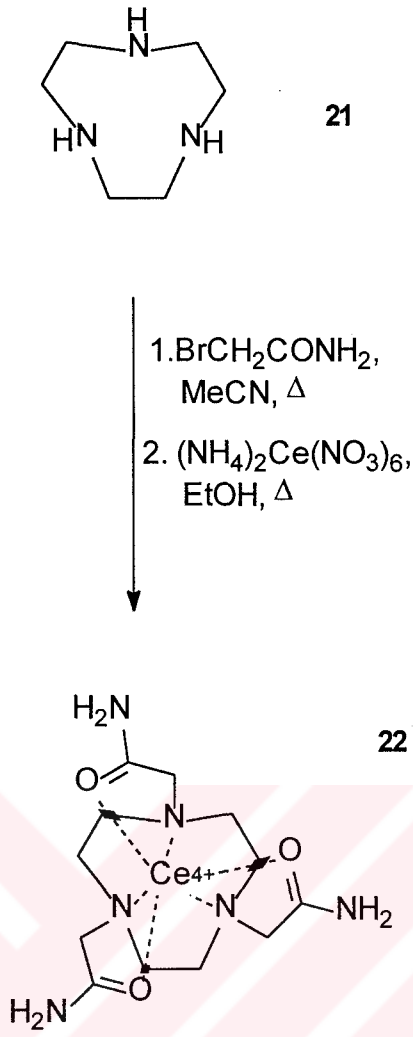


Figure 24: Synthesis of Ce(IV) complex.

2.5. Subcloning of the DNA Insert, HIV-Tat Sequence

Bluescript vector containing the HIV-Tat (HIV transcriptional transactivation gene) sequence, pKSΔPHIV-Tat, was kindly provided by Dr. David Derse, NIH (National Institute of Health). However the vector containing the HIV-Tat had been engineered to remove *Pvu* II sites, which

resulted in destruction of T7 promoter site for transcription. For that reason, subcloning of HIV-Tat to another pBluescript $KS^{(-/+)}$ vector containing T3 and T7 RNA polymerase binding sites, was essential. The subcloning procedure of HIV-Tat sequence was summarized as in Figure 25.

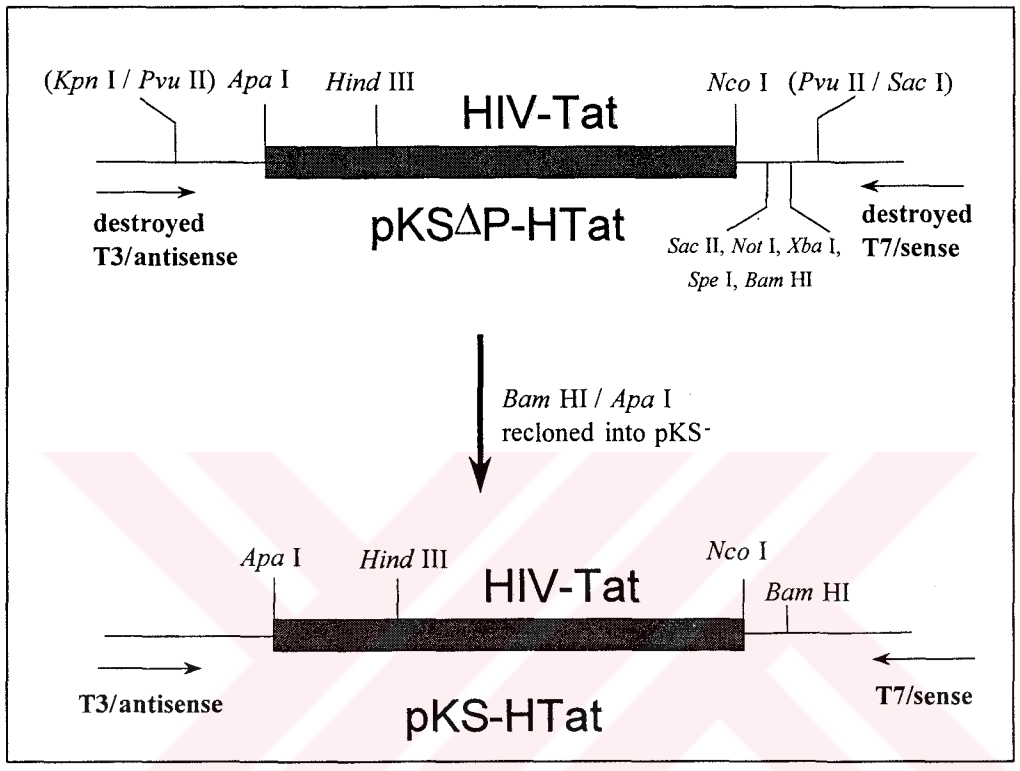


Figure 25: Schematic representation of the subcloning strategy

2.5.1. Preparation of the Vector and the Insert DNA for Subcloning

Both the pKSΔPHIV-Tat plasmid containing the insert HIV-Tat and the vector pBluescriptKS$^{(-/+)}$ (Stratagen, CA) was digested with *Apa* I and *Bam* HI restriction enzymes to generate compatible ends for cloning. 1.125 μL of 5 μL recombinant plasmid was used for the restriction enzyme digestion in 20

μL of reaction mixture. Both plasmids pKSΔPHIV-Tat and pBluescriptKS$^{(-/+)}$ were cut with restriction endonucleases *Apa* I and *Bam* HI in two separate microcentrifuge tubes. 10 μg of DNA, 2 μL10X *Apa* I reaction buffer (MBI Fermentas), 5 μL ddH_2O and 3 μL *Apa* I (MBI Fermentas) restriction enzyme (10 *u*/μL) were pipetted into a clean centrifuge tube, and the reaction mixture was incubated at 37 °C for 4 hrs. The reaction was stopped by heating at 65 °C for 20 seconds. Second restriction enzyme digestion of the vector was performed by adding *Bam H*I enzyme on to the same reaction mixture: 1 μL of *Bam H*I reaction buffer (MBI Fermentas), 6 μL ddH_2O and 3 μL *Bam H*I enzyme (MBI Fermentas) were used for the second enzyme digestion. The reaction was prepared for agarose gel electrophoresis by adding 5 μL of 6X gel loading buffer (MBI Fermentas).

2.5.1.1.Band Isolation of the Restriction Fragment DNA, HIV-Tat

ϕX174 phage DNA, *Hinf* I digested as DNA molecular size marker was used to analyze the restriction fragment containing HIV-Tat sequence released from the plasmid pKSΔPHIV-Tat on a nondenaturing polyacrylamide gel electrophoresis (8% acrylamide/bis-acrylamide (38:1), 1 mL 1XTAE buffer (0.4 M Tris-acetate, 10 mm EDTA, pH 8.0), 450 μL 10% ammonium persulfate (APS), 20 μL N,N,N',N'-Tetramethylelenediamine (TEMED)). The midi size polyacrylamide gel electrophoresis apparatus was employed.

The acrylamide/bis-acrylamide gel part that harbor the restriction fragment HIV-Tat of 249 bp was band isolated in PAGE elution buffer (300 μL) (0.5 M Ammonium acetate (NH_4OAc) (Merck), 10 mM Magnesium acetate $Mg(OAc)_2$, (Fisher Scientific Company), 1 mM EDTA (Sigma) pH 8 and 0.1% SDS (Biofluids). The solution was incubated by shaking overnight at 37°C in an eppendorf tube as described in elsewhere (Sambrook *et al.*, 1989). After this process, the solution was saved and ethanol precipitated.

The obtained restriction fragment was dissolved in 10 μL distilled and deionized water and was stored at -20 °C.

2.5.1.2. Band Isolation of the Restriction Fragment of the Vector DNA from the Agarose Gel

The agarose gels were prepared according to the adapted procedure published by Keller and Manak, 1989. All the agarose gels were performed on a medium size slab agarose gel electrophoresis equipment (IBI Corp., CT USA) (14X10 cm). In order to prepare 1% (w/v) agarose gel, 1 gr FMC agarose was added in 100 mL of 1X TBE (Tris-borate) buffer (TBE working solution: 1X, concentrated stock solution (per liter): 5X: 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 M EDTA (pH 8.0) and agarose melted on a hot plate by continual stirring. Then agarose was allowed to cool below 60 °C, ethidium bromide was added to a final concentration of 0.7 μg/mL and poured into the gel tray. After solidification of the gel, it was placed into the electrophoresis chamber containing 1X TBE buffer. The DNA samples were mixed with 1/10 volume 10X loading buffer, and then they were loaded in to the wells. As a molecular weight marker, *Hind* III digested λ DNA (MBI Fermentas), was used. The gels were electrophorezed at a constant voltage of 60-70 Volts for 3-4 hrs. Gel was illuminated with a shortwave ultraviolet transilluminator for band isolation photography (Polaroid Footdyne). The gel is photographed using a high-speed Polaroid film (667, UK), exposing 30 seconds. The lens setting was 4.5.

Individual restriction fragments were identified following agarose gel electrophoresis and the desired DNA band was excised. The double digested vector (*Apa* I/*Bam* HI) was recovered from the gel slice by using a DNA Isolation Kit (MBI Fermentas, Lithvania).

2.5.2. Ligation of the Insert DNA HIV-Tat and the Vector pBluescript$^{(-/+)}$

20 μL ligation reaction was performed in the presence of all the band isolated insert, 249 bp HIVTat sequence and 0.03 μg prepared vector pBSII(KS$^-$), 2 μL 10X ligation buffer (MBI Fermentas), 2μL ddH_2O, and 2 μL T4 DNA ligase (MBI Fermentas), was incubated at 22 °C overnight. Following the ligation reaction, the plasmid DNA was transformed into competent cells of an appropriate host strain.

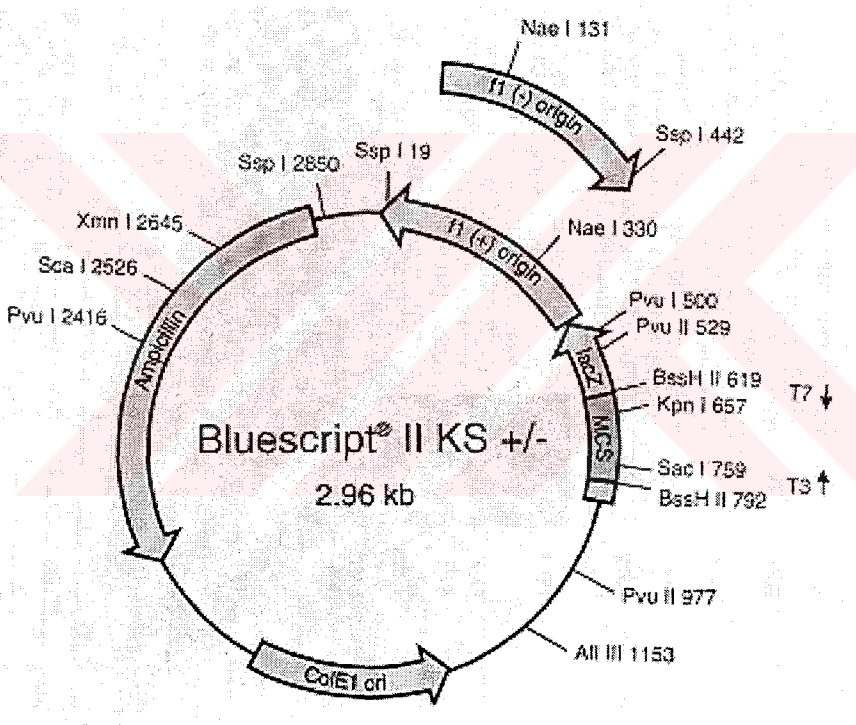


Figure 26: The map of the vector pBSII(KS$^-$) that was used during the subcloning process.

2.5.3. Bacterial Cell Transformation

2.5.3.1.Competent Cell Preparation for Transformation

In the competent cell preparation an *Escherichia coli* cell line, Dh5α, was used. 1mL of overnight-grown *E.coli* (Dh5α) cells were used for inoculation process of 50mL of the LB medium (per liter, 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 1 mL 1 N NaOH). The cell growth was followed by using Shimadzu UV-1601PC Spectrophotometer until the absorbance reading reaching to 0.395 at 590 nm. Subsequently, the grown cells were harvested in a sterile 15 mL polypropylene tubes aseptically by means of a swinging rotor centrifuge (DENLEY BS400, UK) at 4000 rpm for 5 min. Later, the supernatant was decanted and the cells were washed with 10 mL of 50 mM ice-cold sterile $CaCl_2$ solution twice so that the cells become competent. At each time, the cells were gathered as indicated above. Between the washing steps, the cells kept on ice for 10 min. After the second wash step, the cells were suspended in 4 ml 50 mM $CaCl_2$. Following 48 hrs storage at 4 °C to increase the efficiency of transformation, the cells became ready for transformation process (Sambrook *et al.*, 1989).

2.5.3.2.Transformation

The prepared competent cells were used in the transformation process of pKSHIV-Tat subclone.

On 200 μL competent cells, 10 μL of the ligation product was added and gently mixed by inverting the tube up and down. The mixture was kept on ice for 30 minutes and immediately put in a 42 °C incubator for exactly 90 seconds (without shaking) and then rapidly put on ice and incubated for 2-3

min. To the transformants 800 μL prewarmed LB medium was added and they were incubated at 37 °C for 45 min for β-lactamase expression. Finally, transformation products were spread (50μL, 100μL and 300μL) on to LB agar plates (per liter, 10 g tryptone ,5 g yeast extract, 5 g NaCl,1 mL 1 N NaOH, 15 g agar) containing 40 μL (40 mg/mL) X-Gal (Sigma), 10 μL 200 mg/mL IPTC (D-thiogalactopyranoside, Life technologies, Inc.) and 80 μg/μL Ampicillin (Sigma). Liquid LB medium was solidified with agar. For the LB plates, the agar was autoclaved together with the other ingredients of the medium. The agar was cooled to about 50 °C and added to other ingredients like antibiotics if necessary. Finally, the medium was poured into sterile disposable petri dishes (plates) and allowed to solidify. The colonies were left growing for 12-16 hrs at 37 °C in incubator.

The white and light blue colonies were picked up by the help of toothpick and grown in 2 mL LB medium. Mini plasmid preparations were performed according to the literature procedures (Kraft *et al.*, 1988).

2.5.3.3.Selection for Transformants

The conditions to select transformants were standard ampicillin selection and blue/white screening. In order to select the transformants, the transformed Dh5α cells were plated on LB plates containing 100 μL/mL of ampicillin, IPTC and X-Gal and then incubated overnight at 37 °C for the blue/white screening. In the preparation of plates containing X-Gal and IPTC, 20 μL of X-Gal and 100 μL IPTC was spreaded and allowed these components to absorb for 30 min. at 37 °C prior to plating cells.

2.5.4. Isolation of Plasmid DNA

2.5.4.1.Plasmid Preparation by Triton Lysis

5 mL LB medium containing selective agent (100 μg/μL), ampicillin, was inoculated with a single colony of an *E. coli* strain Dh5α containing pKSHIV-Tat. Then it was further grown at 37 °C with vigorous shaking overnight.

500 mL LB medium containing 100 μg/μL of ampicillin was inoculated with the overnight grown 5 mL culture. Later, the culture was grown at 37°C with shaking overnight. Cells were collected by centrifuging 5 min. at 5000 rpm (swinging rotor centrifuge, DENLEY BS400, UK), 4 °C.

Pellet was resuspended from a 500-mL culture in 5 ml sucrose/Tris/EDTA solution (50 mM (Merck) glucose, 25 mM Tris·Cl (Carlo Erba), pH 8.0, 10mM EDTA, (Sigma) autoclaved and stored at 4 °C) and transferred to appropriate centrifuge tube. 1.5 mL of 10 mg/mL hen egg white lysozyme in 25 mM Tris·Cl, 2 mL o.5 M EDTA, and 25μL 10 mg/mL DNase-free Rnase (New England Biolabs) were added to the same tube and standed for 15 minutes on ice. 2.5 mL Triton lysis solution (3% (v/v) triton X-100, 200 mM EDTA, pH 8.0, 150 mM Tris·Cl, pH 8.0, stored indefinitely at 4°C) was overlayed and then mixed gently but thoroughly by inversion. At the end of those processes The tube was let stand 20 min at 4 °C.

This solution should not be vortexed or shaken at all, since that would shear chromosomal DNA and prevent it from precipitating in the next step. The solution will become extremely viscous as the cells lyse. Streaks of opaque material will be visible and may remain throughout the incubation

The solution was centrifuged 70 min at 40,000Xg (17,000 rpm in Beckman JA-17), 4 °C. The supernatant was decanted carefully to a clean centrifuge tube

Contaminating plasmid DNA with gelatinous pellet should be avoided. The pellet contains chromosomal DNA and cellular debris. The pellet may detach from the bottom of the tube. The supernatant was extracted 1:1 buffered phenol/chloroform, then with 24:1 chloroform/isoamyl alcohol. 0.6 volume isopropanol was added to the final aqueous phase and let stand 10 min at room temperature. The plasmid was obtained in the form of pellet by centrifuging 10 minutes at 15,000Xg at room temperature. The pellet was washed with 2 mL of 70% ethanol. Then it was centrifuged briefly to collect the pellet. The pellet was dried by aspirating the ethanol at room temperature.

2.5.4.2.PEG Precipitation of the Plasmid DNA

The pellet was suspended in 2 ml TE buffer (10 mM Tris·Cl, pH 8.0 and 0.1 mM EDTA, pH 8.0. The solution was autoclaved at 120 °C for 20 min) and 0.8 ml of PEG (30% (w/v) PEG 8000, 1.6 M NaCl,) solution was added. This mixture was incubated 15hrs at 0 °C. The plasmid DNA was recovered by centrifugation at 10,000Xg for 20 min, 4 °C. The pelleted plasmid DNA was dissolved in 1 mL TE buffer. The plasmid DNA was ethanol precipitated using 3 M sodium acetate, pH 5.5. The resulting pellet was dissolved in 1ml ddH_2O.

2.5.5. DNA and RNA Concentration Determination

Isolated plasmid DNA or RNA samples were 1:200 diluted with ddH_2O to 1mL and absorbance data were recorded at 280 nm and 260 nm. Nucleic acids absorb light at 260 nm. On the other hand, proteins absorb UV light at 280 nm and the ratio of A_{260}/A_{280} (1.8-2.0) is used to evaluate the purity of the DNA or RNA samples. The concentration of DNA and RNA were determined from the absorbance at 260 nm (1.0 OD_{260}=50μg/mL for DNA, 1.0 OD_{260}=40μg/ml for RNA).

2.6. Visualization

2.6.1. Silver Staining

The adhered gel to the gel plate *via* band silane was transferred to a plastic tray, and immersed into the fixing solution (an aqueous solution of 10% (v/v) ethanol and 5% (v/v) acetic acid) for 6 minutes. The fixing solution was replaced with staining solution (an aqueous solution of 1% $AgNO_3$) and the gel was incubated for 20 minutes. The gel was rinsed once with double distilled water and then was placed in developing solution (an aqueous solution of 1.5% (v/v) NaOH and 0.1% (v/v) formaldehyde (should be added prior to use). The gel was soaked into developing stop solution (0.75% (w/v) aqueous solution of Na_2CO_3) for 20 minutes to stop further development with dark background, after the bands become noticeable. At the end of the silver staining procedure, the gel was scanned.

2.6.2. Visualization of Radioactively Labelled Samples

Sequencing reaction products were electrophorezed on 6% denaturing polyacrylamide gel (for 100 mL: 5.7.g of acrylamide; 0.3 g of bisacrylamide; 48 g of urea; 10 mL of 10X TBE; the volume was completed to 100 mL with ddH_2O; 600 μL of APS; 20 μL of TEMED) at 50 watts. The gel was transferred on to a Whatman paper (3MM), dried on gel drier (Slab Gel Drier SGD2000) and exposed to X-ray film (Kodak Biomax-MR, USA) for 1 week at room temperature. The films were developed at METU Health Center.

2.7. Sequencing of Subcloned HIV-Tat

The plasmid to be sequenced is pKSHIV-Tat, containing the 249-bp fragment. The primers used to sequence the HIV-Tat are T3 and T7 primers, obtained from Promega Biotec. The primers are at a concentration of 10 ng/μL. The Sequenase kit 2.0, including Sequenase DNA is available from United States Biochemical (Cleaveland, OH). The isotope used [α-^{32}P]dATP was purchased from Institute of Isotops Co., Ltd., Hungary. The sequencing electrophoresis was performed using a Stratagene Base Ace Vertical Sequencing Apparatus equipped with 37.5 cm wide plates and 0.4 mm spacers and shark tooth combs. Kodak Biomax-MR, USA was used in the exposure process.

DNA sequencing was performed on one of the clones, which were thought to be candidate containing the insert of interest based on the restriction mapping on 10% polyacrylamide gel. The plasmid DNA was prepared for sequencing by using Qiagen Plasmid Isolation Kit to obtain a pure plasmid DNA, which is favourable for DNA sequencing reaction. The DNA was dissolved in 60 μL distilled deionized H_2O. Concentration of the DNA was 0.375 μg/μL. 10 μL of the DNA sample was taken and the volume

was completed to 20 μL with the addition of 10 μL ddH_2O. 2 μL of freshly prepared solution of 2 N NaOH, 2mM EDTA is added, mixed well, and incubated 5 min at room temperature. On ice, 8 μL ddH_2O and 3 μL 3 M sodium acetate are added sequentially and mixed. 75 μL ice cold 100% ethanol is then added and the sample is precipitated at -20°C for 20 minutes. DNA was collected by centrifuging at 4 °C for 5 min, the supernatant was removed, the pellet was rinsed with 200 μL ice cold 70% ethanol, the tube was spun an additional 2 min at 4 °C, and supernatant was discarded. The sample was dried at room temperature (Kraft *at al.*, 1988). DNA was sequenced by the dideoxy chain termination method of Sanger.

The continuing steps was the modifications of the sequenase protocol: 1 μL of 10 ng/μL the T3/T7 sequencing primer, 2 μL of 5X Sequenase Sequencing Buffer, and 7 μL distilled deionized H_2O are added to the dried denatured DNAs. The samples are mixed and incubated at 37 °C for 30 minutes. The tubes containing 2.5 μL of one of the Termination Mixes (ddGTP, ddATP, ddTTP, ddCTP) were prepared. These tubes, appropriately labelled G, A, T, or C, are pre-warmed at 37 °C. The 1 μL of Sequenase enzyme was diluted to 1:8 with 6.5 μL of enzyme dilution buffer and 0.5 μL of pyrophosphatase enzyme was added to this mixture. The labelling mix was diluted to 1:5 with ddH_2O. The followings were added to each reaction mixture: 1 μL 0.1 M DTT, 2 μL Labelling Mix dilution, 1 μL [^{32}P]dATP, and 2μL diluted Sequenase. These labelling reactions were then incubated at room temperature for 5 min. 3.5 μL of labelling reaction was transferred to each of four termination mix tubes and incubated at 37 °C for 5 min. The reactions were stopped by heating at 94 °C for 1 min in the presence of 4 μL stop solution.

Before being loaded onto a sequencing gel, the samples were heated at 94°C in a termocycler for 2 min and then quickly transferred to ice. The tubes are spun briefly in a microcentrifuge and 2.5 μL of sample was loaded per lane on the sequencing gel. The sequencing gel was run at 50 watts.

2.8. *In vitro* Transcription

The transcription reaction was performed under conditions, which exclude contamination of the reaction mixture with RNases. The reaction mixture was prepared at room temperature, since the RNA can precipitate in the presence of spermidine at 4 °C. The pBSHIV-Tat template DNA (10μg) was linearized with the restriction endonuclease *Apa* I (20 units) in the presence of reaction buffer B^+ (10 mM Tris·Cl, pH 7.5), 10 mM $MgCl_2$ and 0.1 mg/mL BSA) 2μL of reaction mixture of final 20μl reaction volume. The reaction was proceeded for 5 hrs at 37 °C. At the end of the reaction, all of the digested DNA and a size marker λ DNA */Hind* III were loaded to the 1% agarose gel. The size of restriction enzyme digested DNA was determined *via* a size marker λ DNA */Hind* III cut and then, excised. Agarose slices containing the *Apa* I cut plasmid DNA was isolated. The following reaction mixture was prepared: 5X transcription buffer (10 μl) 10 mM 4 rNTP mix (10 μl, 2.0 mM final concentration), linearized template DNA (1μg), ribonuclease inhibitor (50 u), T7 RNA polymerase 30 u, deionized water (nuclease free) up to 50μl. This reaction mixture was incubated at 37 °C for 2hrs. The reaction was stoped by cooling the reaction mixture at -20 °C.

Under the conditions described above, 0.250 μg RNA per 1 μg DNA template was obtained. (Promega Protocols).

2.9. Radioactive Labelling of the Synthetic 30-*mer* RNA

The synthetic 30-*mer* RNA was labeled by using [γ-^{33}P]-dATP, which was purchased from Institute of Isotops Co., Ltd., Hungary. The following reaction mixture was prepared for 5'-end labelling process: 50 pmol of 30-*mer* RNA, 2 μL of 10X reaction buffer (MBI Fermentas), 0.5 μL of [γ-^{33}P]-

dATP, ddH_2O up to 19 μL and 1 μL of T4 polynucleotide kinase (1 μL) (MBI Fermentas). The reaction mixture was incubated at 37 °C for 30 min (Sambrook *et al.,* 1989).

2.10. Cleavage Reactions of the Synthesised Artificial Nucleases

2.10.1.tRNA Hydrolysis by 1,4,7-Tris(carbamoylmethyl)-1,4,7-triaza cyclononane-Ce^{IV} Complex

tRNA hydrolysis was performed at pH 7.4 HEPES buffer (50 mM). In this experiment, yeast $tRNA^{phe}$ (0.13 μg/μL) was treated with either 5 mM or 0.5 mM Ce(IV) complex. Aliquots were taken at 2 hrs intervals and the bands were separated in a 0.4 mm 10% non-denaturing polyacrylamide gel. The bands were observed after silver staining. The gel was then scanned and analyzed.

2.10.2.HIV-Tat mRNA Cleavage Reaction by an Oligo DNA Appended TREN Molecule

The synthesized mRNA was used for the cleavage reaction experiments of the TREN conjugated 12-*mer* DNA. TREN-conjugated 12-*mer* DNA was prehybridized to the complementary mRNA of HIV-Tat in HEPES buffer (20mM pH 7.4) by a heat denaturation (60°C, 10 min) and room temperature cooling (22°C, 10 min) process. HIV-Tat target mRNA hydrolysis by oligo-TREN conjugate was carried out at pH 7.4 with Tris·Cl buffer (0.020 mol dm^{-3}). The HIV-Tat mRNA (0.125 μg in the reaction medium) was treated with oligo-TREN conjugate (200 pmol). Reactions were analyzed by polyacrylamide gel electrophoresis (6% polyacryamide, 7M urea,

Tris-Borate-EDTA running buffer (TBE)). RNA bands were visualized by silver staining.

2.10.3.The Hydrolysis Reaction of 30-*mer* RNA with the Oligo DNA Tethered La^{3+}-Complex

The oligo DNA tethered to the La^{3+}-complex was used in the hydrolysis reaction of 5'-end labelled synthetic 30-mer RNA molecule. The hydrolysis reaction was carried out at pH 7.4 HEPES buffer (20 mM). 2 pmol/μL of 5'-end labelled synthetic 30-mer RNA was treated with 0.03 μg/μL, 0.150 μg/μL, and 0.3μg/μL of the oligo DNA tethered La^{3+}-complex at 37 °C for 18 hrs. Then, the bands were separated in a 15% denaturing polyacrylamide gel. The sequencing gel was run at 50 watts.

CHAPTER III

RESULTS AND DISCUSSION

Compounds capable of cleaving phosphodiester bonds in nucleic acids under physiological conditions are important in drug design, and as tools in molecular biology.

Lanthanide ions and their complexes are very active for the hydrolysis of phosphodiesters. In this thesis, the metal complexes which show catalytic behavior in RNA transesterification at 37 °C and pH 7.4 have been synthesised and their hydrolytic activity have been tested. For the testing purposes, the RNA model,HPNPP, was synthesized and used successufully. These lanthanide complexes which have promoted the transesterification of RNA model phosphodiester compounds were robust toward metal ion release in solution, a property crucial to *in vivo* applications.

One of the most appealing applications of RNA transesterification catalyst is the possibility of forming more efficient antisense nucleotides by attachment of a catalytic cleaving group. It is noteworthy that an oligoamine, in this study tris(2-aminoethyl)amine (TREN) has been used as the catalytic site for the sequence-specific artificial ribonucleases. Sequence-selective RNA scission was accomplished by the attachment of this oligoamine to an oligo DNA. The simplicity of the idea and stability of the TREN conjugate are undoubtedly advatageous for the purpose.

3.1. Hydrolytic Activity of the Cerium Complex of 1,4,7-tris (carbamoylmethyl)-1,4,7-triazacyclononane (TCMT)

The free base form of the ligand, TCMT, was produced in good yield when excess of triethylamine was used in the reaction mixture. The preparation of the same ligand has been reported previously (Amin *et al.*,1996). Here, a novel Ce(IV) complex (**22**) which is remarkably stable in aqueous solutions at pH 7.4 was synthesized. The hydrolytic activity of the complex was studied using the phosphodiester model compound 2-hydroxypropyl-p-nitrophenylphosphate (HPNP, **8**).

It is likely that the hydrolysis proceeds *via* an intramolecular attack by the hydroxide ion coordinated to Ce(IV) toward the phosphate bound to the same centre. Another coordinated water bound to the Ce(IV) is probably associated with the small pK_a (ca.0) of coordinated water (the corresponding value of Ce(III) and other lanthanide (III) ions are 8-9 and thus the concentration of the metal-bound hydroxide ion rapidly decreases with decreasing pH). Furthermore the tetravalent positive charge stabilizes the negatively charged transition state for the phosphodiester hydrolysis (Komiyama, 1995).

Pseudo-first order rate constant for the transesterification (Figure 27) in pH 7.4 HEPES buffer (50 mM) was found to be 0.88 hr^{-1}, that represents a remarkable 7,400-fold rate increase compared to the uncatalyzed reaction (Breslow and Huang, 1991). To best of our knowledge, this is the largest rate acceleration obtained using a lanthanide complex in an additive-free aqueous solution for the hydrolysis of the RNA-model compound **8**. The complex is stable under the hydrolysis conditions, no precipitation of ceric hydroxides were observed for the duration of hydrolysis.

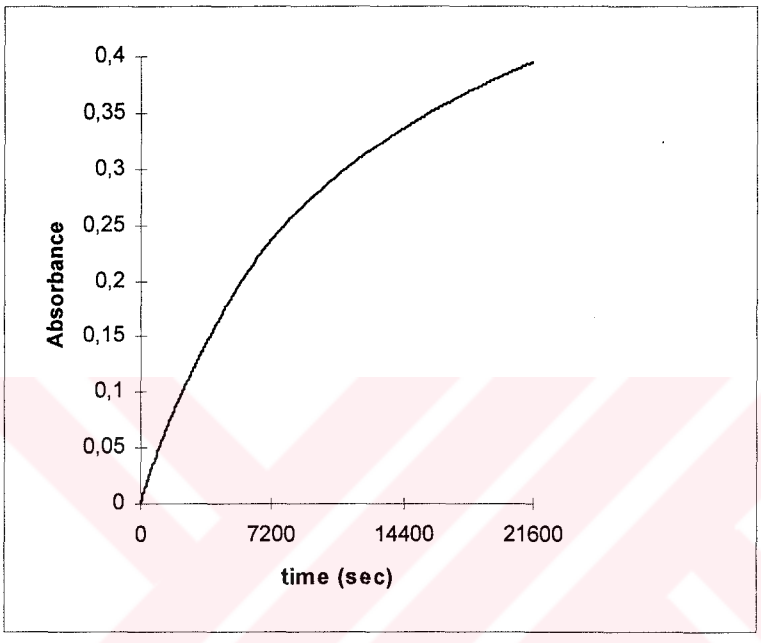


Figure 27: .The transesterification of HPNP ($5X10^{-5}$ mol dm^{-3}) at 25 °C in the presence of 5 mmol dm^{-3} cerium complex of TCMT, as indicated absorption at 400 nm due to the release 4-nitrophenolate ion. The solution was buffered at pH 7.4 with 50 mmol dm^{-3} HEPES.

The activity of the Ce(IV) complex was further studied using yeast $tRNA^{phe}$, as this RNA has been utilized as substrate in a number of previous studies, (Rordorf and Kearns, 1976; Marciniec *et al.*, 1989; Kolasa *et al.*, 1993; Hayashi *et al.*, 1993) where phosphodiester cleaving activity of natural enzymes were mimicked. tRNAs have high degree of secondary structure, and in the earlier studies hydrolytic agents were shown to have differential reactivity at various parts of the RNA structure, mostly attacking the D-loop of the tRNA (Hayashi *et al.*, 1993). Such a result is explained by differing accessibilities of phosphodiester bonds to the metal complex of bulky ligands. Although this may be seen as a way to achieve selectivity, in an artificial enzyme construct, the hydrolytic unit ideally is maximally active, regardless of the secondary structure of the intended substrate; in a real-life situation, targeted RNA segment may have a similar protected structure and less than maximal hydrolysis in those regions would not be desirable. Once a highly active complex is developed, better selectivity could achieved by conjugating the hydrolytic unit to an antisense oligonucleotide (Magda *et al.*, 1994; Magda *et al.*, 1997).

The lanthanide metal complexes of hexaimine macrocyclic ligands L_1 and L_2 hydrolyze the $tRNA^{Phe}$ much more selectively than lanthanide metal ions without the ligands (Figure 28). Compared to those complexes, the cerium (IV) complex that we have synthesized show no sequence specificity. It hydrolyzed the $tRNA^{Phe}$ effectively without exhibiting no base or structure specificity due to the high hydrolytic activity. And also this cerium (IV) complex (**22**) has smaller structure than the complex synthesized by Hayasi *et al.*, 1993. Therefore, the ability of the cerium (IV) complex (**22**) to penetrate further through the secondary structure of the tRNA because of its structure made it more active.

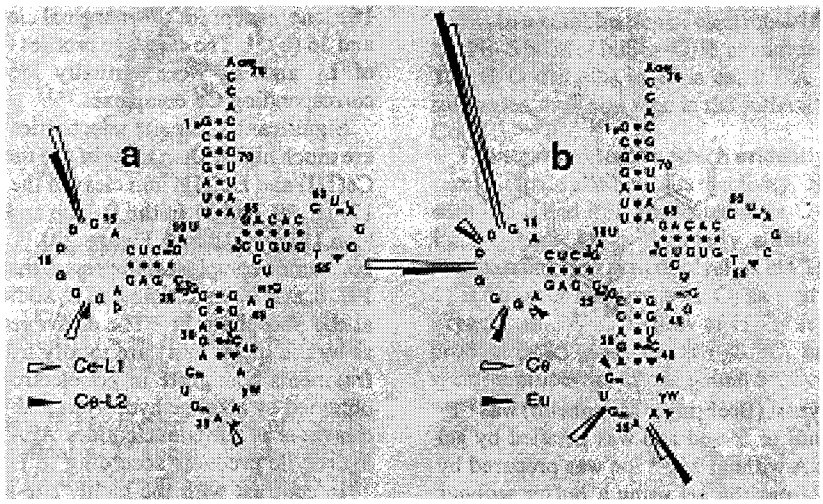


Figure 28. The hydrolysis sites of $tRNA^{Phe}$ for the cleavage by the Ce complexes (**a**) and by the metal ions (**b**). The length of the arrows correspond to the extend of hydrolysis after incubation with Eu and La complexes (Hayashi *et al.,* 1993).

Duplex RNA is less flexible than single-stranded RNA. As a result of this decreased flexibility, the hydrolysis of duplex RNA is inhibited since the attacking 2'-OH must properly orient itself for in line displacement. The binding of Ce(IV)-complex to RNA may alter the orientation of the phosphate backbone, thereby facilitating the nucleophilic attack (Hayashi *et al.,* 1993).

tRNA hydrolysis was carried out at pH 7.4 HEPES buffer (50 mM). In a typical experiment, yeast $tRNA^{phe}$ (0.13 μg/μL) was treated with either 5 mM or 0.5 mM Ce(IV) complex. Aliquots were taken at 2 hrs intervals (Figure 29) and the bands were separated in a 0.4 mm 10% non-denaturing polyacrylamide gel. Unlike other works in the field, we chose to visualize the RNA degradation using a non-radioactive method: the bands were made

visible by silver staining. The gel was then scanned and analyzed. The cerium complex at 5 mM concentration, results in an essentially complete degradation of the tRNA in 6 hrs. It appears even the smaller fragments which are formed are further hydrolyzed to ribonucleoside level. This remarkable hydrolytic activity is not affected by the addition of excess EDTA, further demonstrating that the activity is not due to free Ce(IV) ions. The extent of hydrolysis is dependent both on the time of incubation and on the metal ion coordination; control reactions where RNA was incubated in the absence of Ce(IV)-complex exhibited no RNA degradation. These results confirm that the metal complex alone, not the buffer or adventitious metal ions, is responsible for RNA hydrolysis. The scission proceeds almost uniformly throughout the RNA chain; no specific base preference was perceived. Thus, we have synthesized a complex of high activity towards HPNP and RNA causing total hydrolysis of yeast $tRNA^{phe}$ at neutral pH.

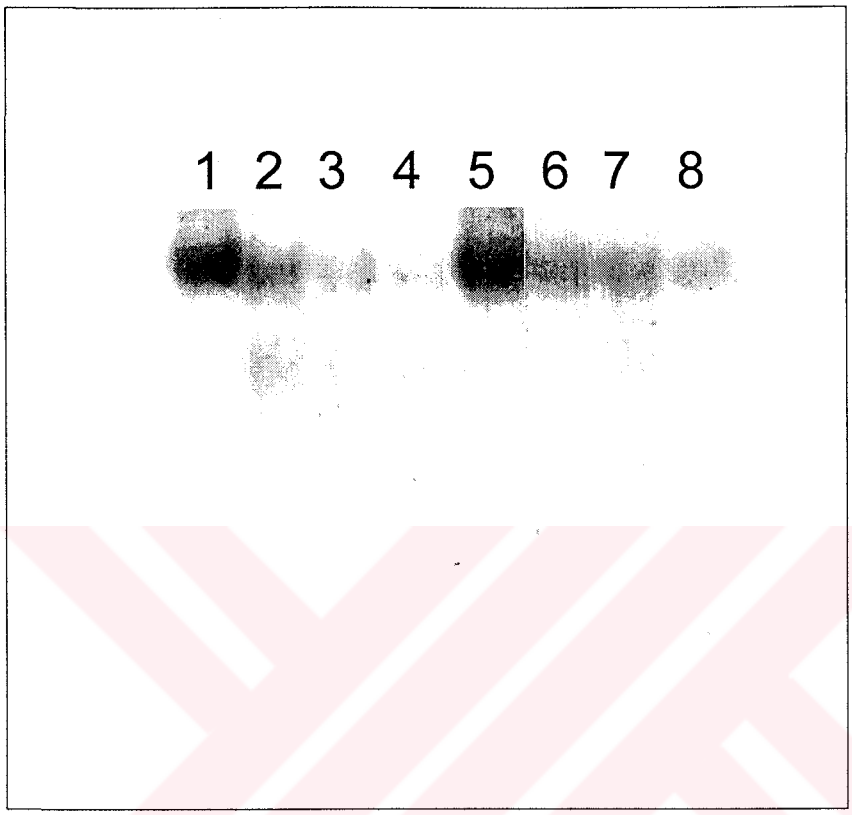


Figure 29: Silver-stained, 10% non-denaturing polyacrylamide gel showing the extent of RNA degradation in the presence of the Ce(IV) complex (**22**). The reaction was carried out at 37 °C in pH 7.4 HEPES buffer (50 mM); yeast $tRNA^{phe}$ (0.13 μg/μL) was treated with either 5 mM (lanes 1-4) or 0.5 mM (lanes 5-8) Ce(IV)-complex. Aliquots were taken at 2 hrs intervals and diluted 10-fold with buffer, and 7 μL of the diluted reaction mixture was applied to the gel. Lane 1 and 5, 0 hrs; lanes 2 and 6, 2 hrs; lanes 3 and 7, 4 hrs; lanes 4 and 8, 6 hrs of reaction.

3.2. Hydrolytic Activity of Lanthanum Complex of NPAC

Lanthanide complexes are particularly impressive in their hydrolytic activity. In fact, certain lanthanide salts are good catalysts of RNA and phosphodiester hydrolysis in general. But to harness their activity and direct it to the desired region of the target RNA/DNA molecule, kinetically stable, yet hydrolytically active complexes that carry a 'handle', a functional group that would allow conjugation to a complementary sequence of DNA is needed. Morrow reported a derivative of cyclen-derived ligand, p-nitrobenzyl-tris(carbamoylmethyl)cyclen (NBAC) (**23**) but its complex with La^{3+} is not stable in aqueous solutions at neutral pH, because one of the donor atoms is lost, making the ligand a heptadentate rather than a octadentate ligand (Amin *et al.,* 1996). In our own approach for the synthesis of a DNA-conjugatable complex, we started with p-nitroaniline, converted it to a chloroacetanilide, reacted it with cyclen, and finally reacted the cyclen derivative with bromoacetamide and formed the complex with lanthanum. The approach has allowed for a direct and scalable synthesis of efficient lanthanide complex from the alkylated cyclen that contains a linking moiety that enable the attachment of an oligonucleotide. This novel octadentate ligand p-nitrophenylcarbamoyl-tris(carbamoylmethyl)cyclen (**28**) forms very stable complexes with a number of lanthanide ions as evidenced by trapping experiments carried out with excess Cu^{2+}.

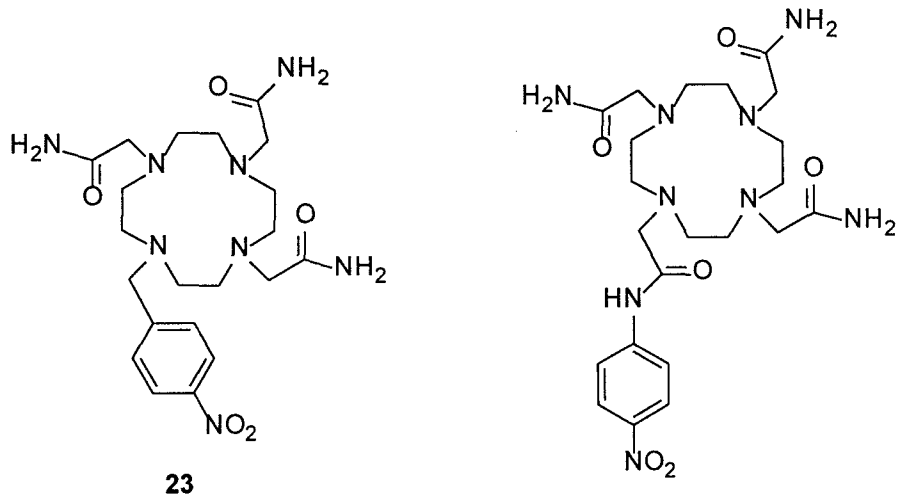


Figure 30*:* Structure of the heptadentate ligand NBAC (**23**) and the novel octadentate ligand, NPAC (**18**).

The hydrolysis of 2-hydroxypropyl-p-nitrophenylphosphate (Figure 31) was studied under pseudo-first order conditions: the hydrolysis reaction was carried out at pH 7.4 buffer (50 mM HEPES) using 0.05 mM of the phosphodiester and 3 mM of the complex. The observed rate constant was 8.0×10^{-2} h^{-1}. The rate acceleration was approximately 700-fold, comparable to that of unmodified TCMC (tetrakiscarbamoylmethylcyclen) complex of lanthanum. Large excess of EDTA did not result in a decrease in the hydrolysis rate confirming that the hydrolysis is not due to free La^{3+}. The bifunctional ligand synthesized exhibits high kinetic stability in neutral aqueous solutions and also as active as the unmodified TCMC complex in phosphodiester hydrolysis. The nitro-substituent is a good handle, and after a reduction to an amino group it reacted with appropriate oligonucleotide derivatives by standard protocols.

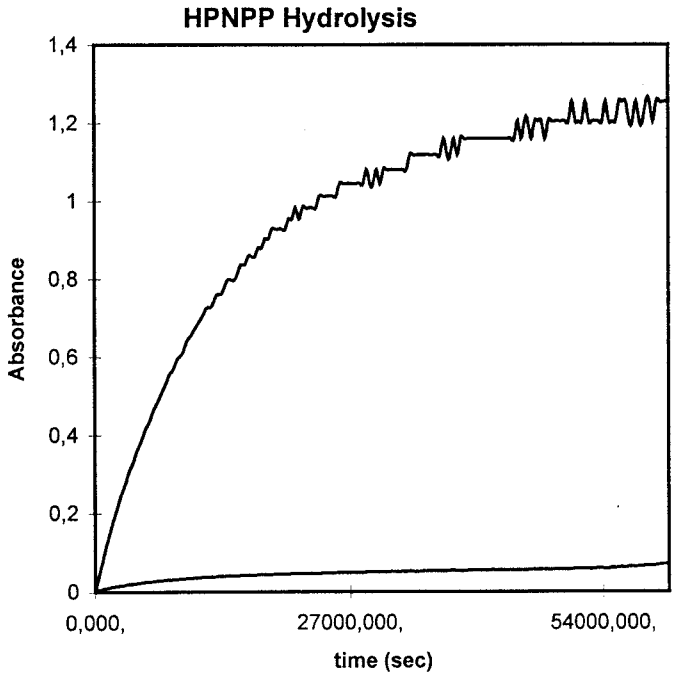


Figure 31: The transesterification of HPNP ($5X10^{-5}$ M) at 25 °C in the presence of 3 mM lanthanum complex of NPAC, as indicated by an increase in absorption absorption at 400 nm due to the release 4-nitrophenolate ion. The solution was buffered at pH 7.4 with 50 mM HEPES. The lower curve is the uncatalyzed reaction.

Oligo DNA has been appended to the La^{3+}-complex. Then, under physiological conditions, hydrolysis of the synthetic 30-*mer* RNA, has been accomplished successfully. It has been observed that no site-specific cleavage has occurred. Highly hydrolytic activity of the La^{3+}-complex led to the total hydrolysis of the synthetic RNA (Figure 32).

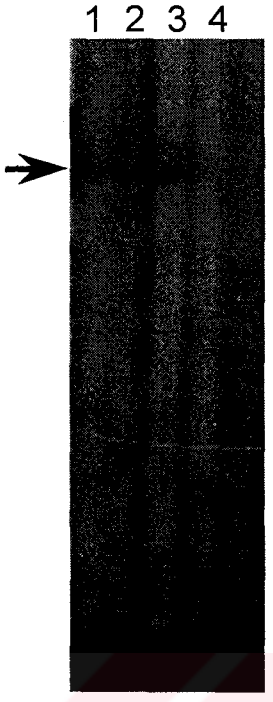


Figure 32: Cleavage patterns of 5'-end labelled synthetic 30-*mer* RNA by the La^{3+}-complex shown by a autoradiography on a 15% denaturing polyacrylamide gel. Lane **1:** Only 2 pmol RNA as control; Lane **2:** 2 pmol RNA was incubated for 18 hrs at 37 °C under physiological conditions; Lane **3:** 2 pmol of the labelled RNA incubated in the presence of 0.03 $\mu g/\mu L$ of La^{3+}-complex, Lane **4:** 0.150 $\mu g/\mu L$ of La^{3+}-complex, Lane **5:** 0.3 $\mu g/\mu L$ of La^{3+}-complex for 18 hrs at 37 °C under physiological conditions.

3.3. Subcloning and Bacterial Transformation

A trans-activating regulatory protein encoding gene HIVTat was subcloned from the vector pKS$_\Delta$PHIVTat into pBluescript KS$^-$. Both vectors

were digested sequentially by *Apa* I and *Bam* HI restriction enzymes. Figure 5 and Figure 6 shows the restriction enzymes digestion of pKS$_{\Delta}$PHIVTat and pBluescript KS^{-} respectively.

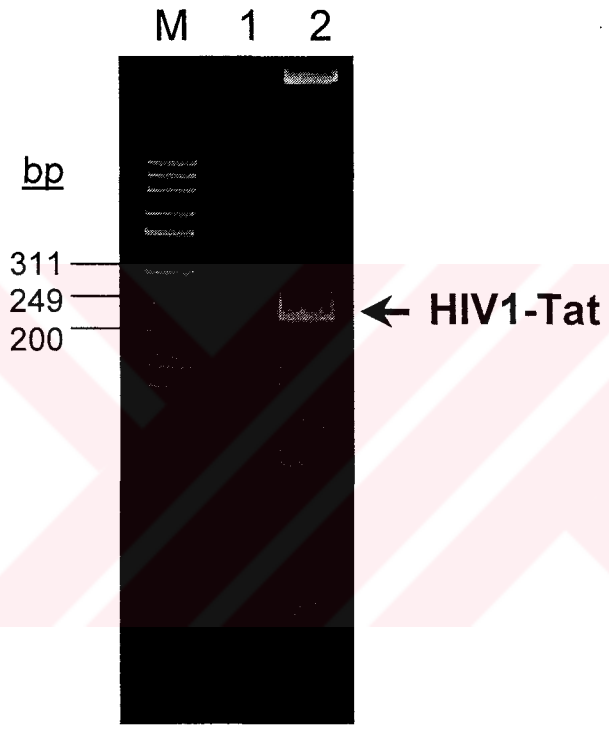


Figure 33: *Apa* I and *Bam* HI digested pKS$_{\Delta}$PHIVTat plasmid carrying the cloned HIVTat fragment of the HIV-1 virus genome in 8% PAGE. **M**: DNA molecular size marker, ϕX174 DNA/*Hinf* I digest; Lane **1**: Empty; Lane **2**: Double digested pKS$_{\Delta}$PHIVTat (*Apa* I/*Bam* HI). Arrow indicates the position of HIVTat DNA sequence.

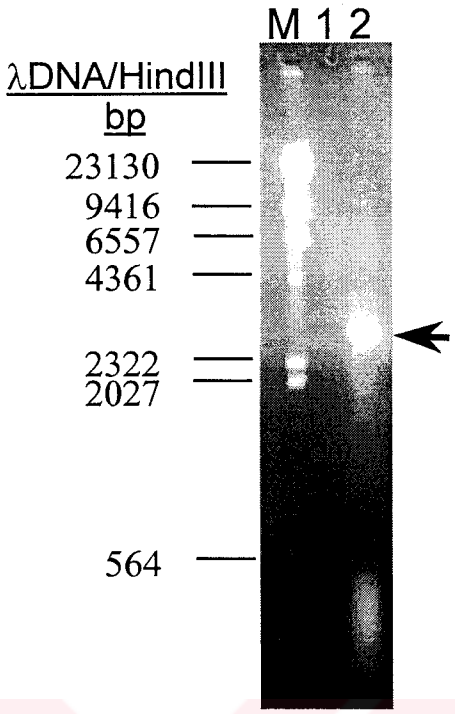


Figure 34: 1% Agarose gel electrophoresis of double digested vector pBluescript KS^-. Lane **M**: *Hind* III digested λ phage marker; Lane **1**: empty; Lane **2**: *Apa* I/*Bam* HI cut cloning vector pBluescript $KS^{+/-}$.

The DNA fragments were isolated from the gels shown in Figure 33 and Figure 34 successively. Outcome of these processes was used for ligation to attain the vector, pKSHIVTat, which was going to use as the template in the trascription stage. The ligation product was transformed into the bacteria by using the way as indicated in the materials and methods part.

3.4. Confirmation of the Cloning of HIV-Tat Sequence into the pBluescript $KS^{+/-}$

Following the transformation, the supercoiled plasmid DNA was harvested according to the standard procedures mentioned about at materials and methods part and characterized by the restriction mapping illustrated in Figure 30. The restriction ezyme digestion reaction of both Bluescript vector and subclone pKSHIV-Tat with the enzyme *Apa* I was followed by the enzyme *Bam* HI. The digestions were successfully released the insert at the expected size. This insert is the HIV-Tat sequence that has been desired to be cloned. The other fragment is a part of the multiple cloning site (MCS) from the vector Bluescript.

Then, in order to be sure about the subclone, the subcloned HIVTat sequence was sequenced. The pKSHIV-Tat plasmid DNA was subjected to the sequencing reactions (according to the procedures in Materials and Methods part). The scanned image of typical sequencing autoradiogram of the results was illustrated in Figure 34. Nearly, the entire sequence of the HIV-Tat has been determined from the autoradiogram (Figure 35).

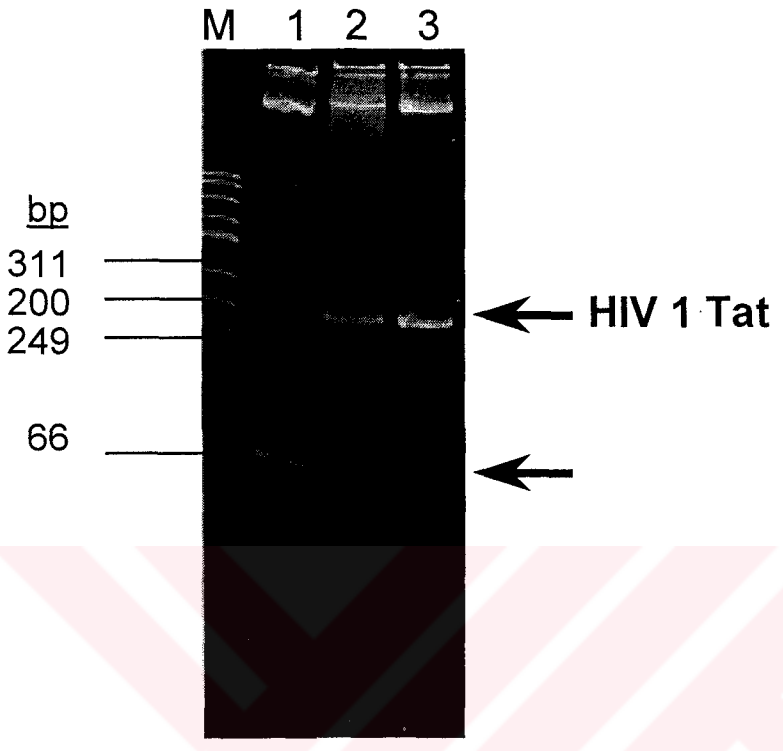


Figure 35: Analysis by 10% PAGE electrophoresis of the cloned plasmid Bluescript vector. Lane **M**: a marker revealing the restriction pattern ϕX174 by *Hinf* I; Lane **1**: *Apa* I/*Bam* HI cut Bluescript vector; Lane **2,3**:*Apa* I/*Bam* HI cut HIVTat cloned Bluescript vector. Bottom arrow indicates the *Apa* I/*Bam* HI deletion product from MCS.

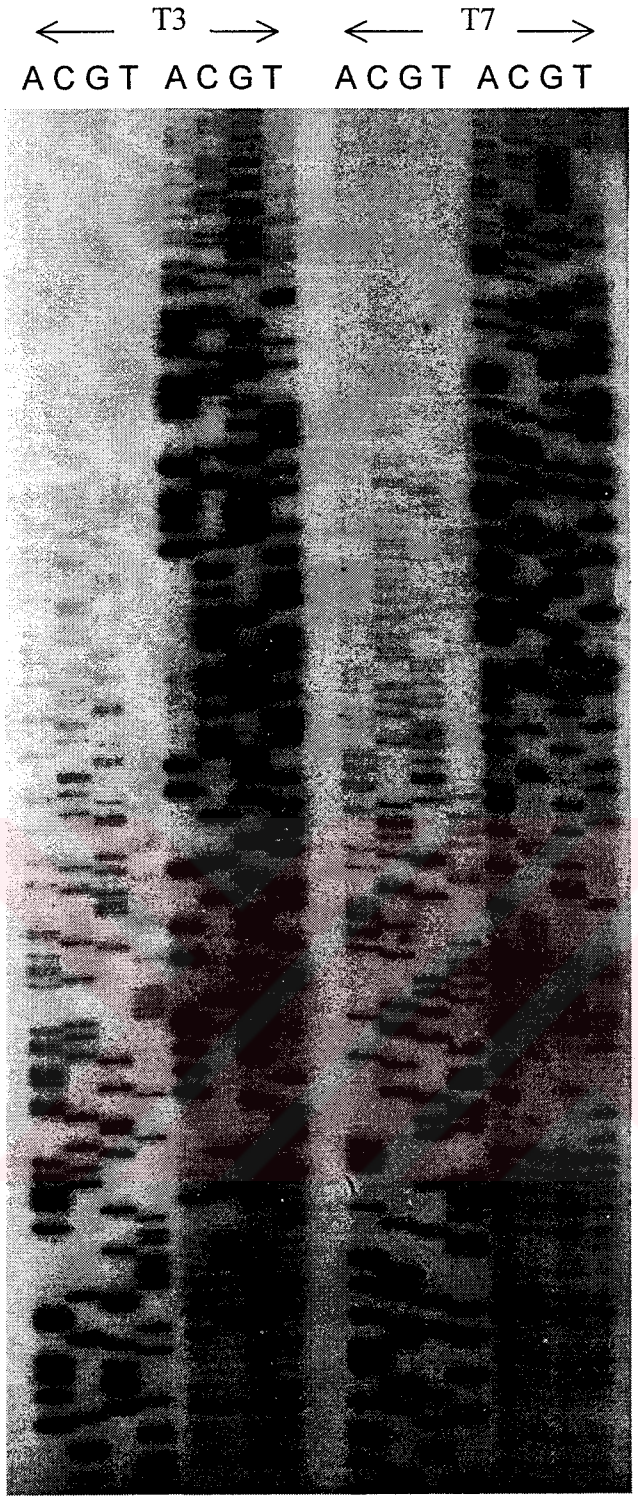


Figure 36: Sequencing reactions of the insert of pKSHIV-Tat.

5'...	TTA	CTG	CTT	TGA	TAG	AGA	AGC	TTG
ATG	AGT	CTG	ACT	GTT	CTG	ATG	ATC	TCT
TCG	TCG	CTG	TCT	CCG	CTT	CTT	CCT	TAC
GTA	GGA	GAT	GCC	TAA	GGC	TTT	TGT	TAT
GAA	ACA	CAG	CTG	GCA	ATG	AAA	GCA	ACA
CTT	TTT	ACA	ATA	GCA	ATT	GGT	GCC	ATG
CAG	TTT	TAG	GCT	GAG	...3'			

(a)

5'...	ACC	AAT	TGC	TAT	TGT	AAA	AAG	TGT
TGC	TTT	CAT	TGC	CAG	CTG	TGT	TTC	ATA
ACA	AAA	GCC	TTA	GGC	ATC	TCC	TAC	GTA
AGG	AAG	AAG	CGG	AGA	CAG	CGA	CG...	...3'

(b)

Figure 37: Read sequence of HIV-Tat DNA in the subcloned plasmid DNA **(a)** from T3 promoter site, and **(b)** from T7 promoter site has been confirmed that the cloning process has been performed successfully

For the synthesis of the HIV-Tat mRNA, the plasmid was linearized with *Apa* I and then band isolation was performed from 8% agarose gel. And

then the RNA was synthesized with T7 RNA polymerase. There was a common difficulty in the RNA isolation, since most ribonucleases are very stable and active enzymes require no cofactors to function. Eventhough, all the required precaution was taken, recovery of the synthesized mRNA from the reaction mixture would not have been possible at every reaction for synthesis.

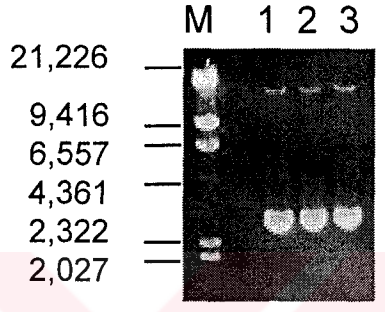


Figure 38. *Apa* I digested subclone pKSHIV-Tat carrying the cloned HIV-Tat sequence for *in vitro* mRNA preparation. Lane **M**: DNA molecular size marker, *Hind* III digested λ phage; Lane **1, 2, 3**: *Apa* I digested pKSHIV-Tat plasmid.

The RNA substrate, prepared by in vitro transcription from HIV-Tat DNA sequence was treated with oligo appended tren molecules. When the complementary 12-*mer* sequences of oligo-tren conjugate and the corresponding sequence in the target RNA molecule form a double helix, the hydrolytic power of tren molecule was localized in the vicinity of the phosphodiester linkages which was hydrolyzed (Figure 34).

3.5. Oligo-TREN Activity on the Target RNA Sequence of HIVTat

An *in vitro* selection procedure was used to develop a sequence-specific artificial enzyme that can be made to cleave almost any targeted RNA substrate under simulated physiological conditions. The enzyme is comprised of a catalytic domain and a substrate recognition moiety.

The 5'-end of a 12-mer DNA oligomers was modified by attaching TREN molecule via a phosphoramidate linkage. The oligomer was designed to be complementary to the HIV-Tat protein-coding region. The hydrolytically active part functions in general acid/general base mode to accelerate the phosphodiester hydrolysis and thus cleave the targeted sequence.

The RNA hydrolysis by the TREN-DNA conjugate was carried out at pH 8 (1mmol dm^{-3} Tris buffer) and 37 °C in the presence of EDTA (1mmol dm^{-3}) and was analysed by 6% denaturing polyacrylamide gel electrophoresis. RNA substrate, prepared by in vitro transcription from HIVTat DNA sequence was cleaved by the artificial enzyme. This process generated almost two products of the expected size, as judged by their electrophoretic mobility in comparison to the standards. The products were separated on a 6% denaturing PAGE containing 7M urea.

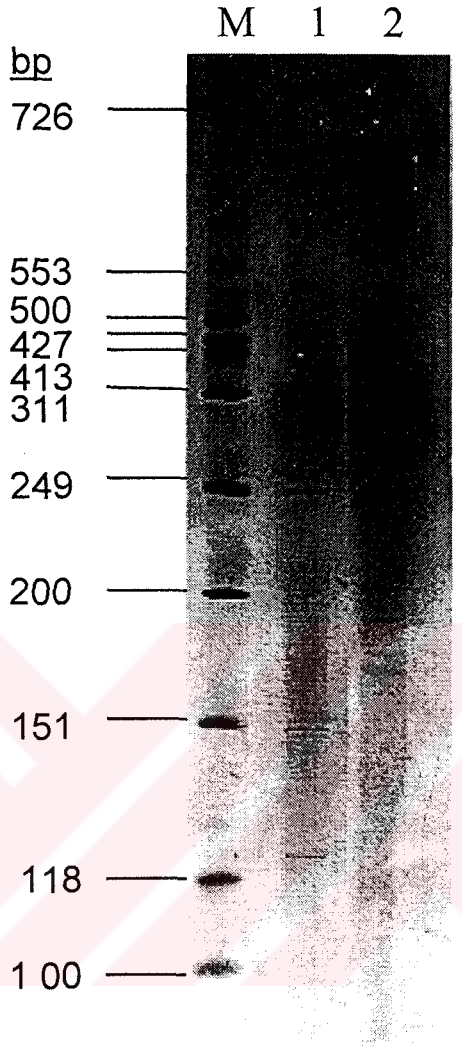


Figure 39: Analysis of mRNA cleavage by silver stained 6% denaturing PAGE. Lane **M**: ΦX174 DNA/*Hinf* I as DNA molecular size marker; Lane **1**: 276-base HIV-Tat mRNA (0.125 μg) was annealed to the 200 pmol antisense oligo-tren and allowed to incubate for 8 hrs at 37 °C in 20 mM, Tris.Cl pH 7.4 buffer treated HIV-Tat mRNA; Lane **2**: HIVTat mRNA (0.125 μg) in the absence of antisense oligo-tren as control.

CHAPTER IV

CONCLUSION

In this study, the design, preparation and activity of some functional nuclease mimics have been accomplished.

An octadentate ligand based on the saturated 1,4,7,10-tetraazacyclododecane-ring skeleton was synthesized as a kinetically robust complex. This macrocyclic lanthanum complex which is conjugated to the oligonucleotide for the sequence specific-cleavage is important within the context of antisense technology.

The activity of the Ce(IV) in the hydrolysis reactions is nothing short of spectacular. For this reason Ce(IV) was used as the catalytic center one of the of the artificial enzyme used in this study. In conclusion, yeast t-RNA^{Phe} and model phoshodiester compound HPNPP have been hydrolyzed efficiently at pH 7.4 and 37 °C nonenzymatically by use of the Ce(IV)-complex. Hydrolysis has been achieved in totally homogeneous solutions. Significantly, Ce(IV)-complex cleaves both single- and double-stranded RNA. Therefore, it could be useful in RNA footprinting. A potential use of Ce(IV)-complex for artificial regulation of cell functions can be envisioned.

Sequence-selective and hydrolytic artificial nucleases were prepared by the attachment of the complexes to the DNA-oligomers to hydrolyze the HIVTAt RNA sequence. The RNA scission was achieved by the attachment of the oligoamine molecule, tren (tris(2-aminoethyl)amine), to the synthetic

DNA oligomer. Tren molecule was effective in the hydrolysis of the target HIVTat mRNA due to intramolecular cooperation of two amino residues. The choice of the sequence and length of the DNA oligomer was arbitrary, and thus any phosphodiester linkage in the nucleic acid molecule can be hydrolyzed with a desired sequence-specificity. A reagent that prevents a specific single-stranded RNA from participating in protein synthesis *in vivo* would obviously have many important applications.

Matching of the present artificial enzymes with current biotechnology is straightforward. These synthetic enzymes totally free from contamination by natural enzymes and other biomaterials. In the near future, artificial nucleases will be used widely both *in vitro* and *in vivo*, greatly contributing to relevant fields.

These investigations targeting specific RNAs for degradation are important steps toward the goal of developing drugs for gene therapy.

This study combines the power of organic synthesis with the potential of the antisense technology.

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  - electro elution, *etc*...
- DNA marker preparation (pBR322 / Msp I)
- cloning
  - sub-cloning of HIVTat gene from a vector into $pBSKS^{+/-}$
- *E. coli* competent cell preparation
- transformation of *E.coli* strains, XL1 Blue and DH5α.
- *in vitro* RNA synthesis/ run off transcription
- all kinds of RNA/ DNA/protein gel electrophoresis
  - PAGE
  - SDS-PAGE
  - agarose gel
  - denaturing polyacrylamide gels for sequencing and other purposes
- non-radioactive (other than Ethidium Bromide) staining of DNA and RNA gels
  - cyber-green detection
  - silver staining
- radioactive and non-radioactive labelling of RNA and DNA
  - 5'-end radioactively labelled RNA and DNA
  - *in vitro* radioactively labelled RNA synthesis
  - Dig-dUTP incorporation into RNA
- Southern and Northern blotting and hybridization
- Electroblotting
- PCR
  - PCR primer design
  - amplification of HIVTat DNA
- DNA sequencing/Sanger's method; USB Seq. Kit Version 2.0
- protein isolation
  - invertase from Beaker's yeast
  - taq polymerase from expression vector
- experienced in using UV/Vis Spectrophotometer

2. Synthesis of various ligands for preparation of hydrolytically active metal complexes, preparation of DNA polyamine conjugates, and study of their hydrolytic activities.

3. FTIR studies of the lipid-lipid, lipid-drug interactions in biological membranes

4. Undergraduate Research: Effects of detergents (such as Triton X-100, SDS... which are used in the solubilization of the microsomes) on the electrophoretic movements of the proteins.

Computer Skills

Skilled in the use of windows-based software.

M.S and Ph.D Studies

Subject of Ph.D Thesis: Synthesis and activities of DNA oligomers with appended hydrolytic sites.

Subject of M.S. Thesis: Interaction of vitamin-E with cholesterol containing-phosholipid membranes: An FTIR study.

Awards

Awarded fellowships during the MS and Ph.D. studies by the "Scientific and Technical Research Council of Turkey"

Teaching Experience

Supervised General Chemistry laboratory courses.

Publications

1. Baykal, U., and Akkaya, E.U., "Synthesis and Phosphodiester Transesterification Activity of the La^{3+}- Complex of a Novel Functionalized Octadentate Ligand". *Tetrahedron Letters* **39**: 5861-5864, 1998.

2. Baykal, U., Akkaya, M. S., and Akkaya, E. U. "Remarkable Phosphodiester Hydrolysis Activity of a Novel Ce^{IV} Complex in Neutral Aqueous Solutions" (Accepted for publication in Journal of Molecular Catalysis) 1999.

3. Baykal, U., Akkaya, M. S. and Akkaya, E.U. "A novel Lanthanide Complex with Remarkable Phosphodiester Transesterification Activity and DNA-Conjugatable Functionality, (accepted for publication in Journal of Inclusion Phenomena and Macrocyclic Chemistry), 1999.

4. Severcan, F., Kazanci, N., Baykal, U., Süzer, S., "IR and Tubidity Studies of Vitamin E -Cholesterol-Phospholipid Membrane Interactions"*Bioscience Reports*, **15(4)**: 221-229, 1995.

5. Severcan, F., Baykal, U., Süzer, S., "Vitamin E -cholesterol-DPPC Membrane Interactions in CH_2 Region". *Fresenius Journal of Analytical Chemistry*, **335**: 415-417, 1996.

6. Severcan, F., Baykal, U., Süzer, S., "Interaction of Vitamin E with Cholesterol Containing Model Membranes.", *5th National Biophysics Congress*, , Izmir, TURKEY, S5, September, 23-25, 1993.

7. Baykal, U., Süzer, Ţ., Severcan, F. "Vitamin E Influences the Interaction of Cholesterol with Phospholipid Membranes: An FTIR Study". *NATO ASI, Trafficking of Intracellular Membranes,* Esphino,PORTUGAL, June 1994.

8. Baykal, U., Severcan, F., Süzer, S., "FTIR Studies of Interaction of Vitamin-E with Cholesterol-Containing Liposomes." *6th National Biophysics Congress*, Silivri, Istanbul, TURKEY, B2, September 28-30, 1994.

9. Severcan, F., Kazanci, N., Baykal, U., Süzer, S."Optical Studies of Vitamin E -Cholesterol-Phospholipid Model Membrane Interactions". *23rd Meeting of the Federation of European Biochemical Societies*, Convention Center Basel, SWITZERLAND, P37.2 August 13-18, 1995.

10. Severcan, F., Kazanci, N., Baykal, U., Süzer, S. "IR and Turbidity Studies of Vitamin E -Cholesterol-Phospholipid Model Membrane Interactions". *35th IUPAC Congress,* Istanbul, TÜRKIYE, PHYS.PO36, 14-19 August, 1995.

11. Interaction of Vitamin E with Cholesterol-Containing Phospholipid Model Membranes: An FTIR Study., Master Thesis by Ulku Baykal, in Biotechnology, METU, February, 1995.

12. Baykal, U., Akkaya, E. U., Akkaya, M. S. "The Design and Analysis of Oligonucleotide Conjugates with Hydrolytically Active Functional Groups", *Turkish Biiochemical Society, 13th International Biochemistry Congress*, Antalya, TÜRKIYE, C-105, March 26-30, 1996.

13. Severcan, F., Baykal, U., Süzer, Ţ. "Concentration and Temperature Induced Effects of α-Tocopherol on Cholesterol-Containing Phospholipid Model Membranes". *23rd European Congress on Molecular Spectroscopy*, Budapest, HUNGARY, August 25-30, 1996.

14. Baykal, U., Akkaya, E. U., Akkaya, M. S. "Oligonucleotide with Hydrolytically Active Functional Groups as Potential Antisense Agents", *8th European Congress on Biotechnology*, Budapest, HUNGARY, TU-1116, August 17-21, 1997.

15. Baykal, U., Akkaya, M. S., Akkaya, E. U. "Hydrolytic Scission of RNA by a Cerium Complex", *25th Silver Jubilee FEBS Meeting*, P42.05, Copenhagen, DENMARK, July 5-10, 1998.

16. Baykal, U., Akkaya, M. S., Akkaya, E. U. "Novel Ce(IV) and La(III) Complexes as Efficient Phosphodiester Hydrolyzing Artificial Enzymes" *1st Internationa Conference on Supramolecular Science and Technology*, pp. 190, Zakopane, POLAND, October 3, 1998.