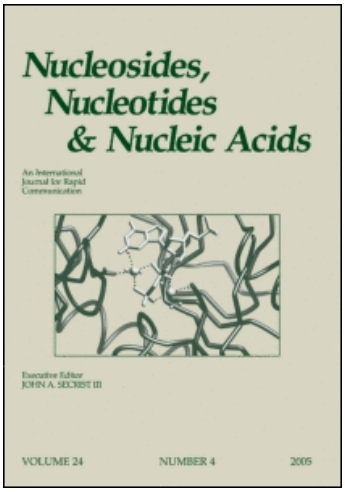

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Sequence-Specific Cleavage of RNA Using Macrocyclic Lanthanide Complexes Conjugated to Oligonucleotides: A Structure Activity Study

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V. NOVEL NUCLEOSIDES AND OLIGOMERS

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1357-1368 (1997)

SEQUENCE-SPECIFIC CLEAVAGE OF RNA USING MACROCYCLIC LANTHANIDE COMPLEXES CONJUGATED TO OLIGONUCLEOTIDES: A STRUCTURE ACTIVITY STUDY

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ABSTRACT. A series of lanthanide complexes were synthesized and conjugated to an oligodeoxynucleotide. Sequence-specific cleavage of a complementary RNA by the conjugates was performed and results are discussed in terms of structure of the complexes.

INTRODUCTION: The sequence specific cleavage of RNA is a theme of current interest, especially in the field of antisense technology.[1-3] The use of metal complex catalysts conjugated to oligonucleotides as artifical ribonucleases is a promising development.[4-11] In particular, some macrocyclic lanthanide complexes have proven effective in this role for the hydrolytic cleavage of RNA.[8-11] We report here the synthesis of a series of lanthanide complexes, their conjugation to an oligodeoxynucleotide and the cleavage of a complementary RNA target. Yields of the cleavage reaction are discussed in terms of structure of the complex, compared to the parent complex.

RESULTS AND DISCUSSION: The use of hexaazamacrocycle (HAM) complexes of the the lanthanides (FIG. 1; complex **I**) as transesterification catalysts for phosphotriesters[12] and RNA[13,14] has been described. This is of relevance in the antisense field because of the ongoing search for efficient artificial ribonucleases - oligonucleotides bearing covalently linked catalyst entities for the sequence specific cleavage of RNA.[15,16] Three features that made these complexes of special interest to us, with this application in mind, were the reported high kinetic inertness of the complexes coupled with a straightforward synthesis and a catalytic action in the cleavage reaction.[13] Thus, we used a carboxylic acid-bearing diacetyl pyridine to synthesize a monofunctionalized HAM-La complex (**II**) which was covalently linked to the 5'-end of an oligodeoxynucleotide.

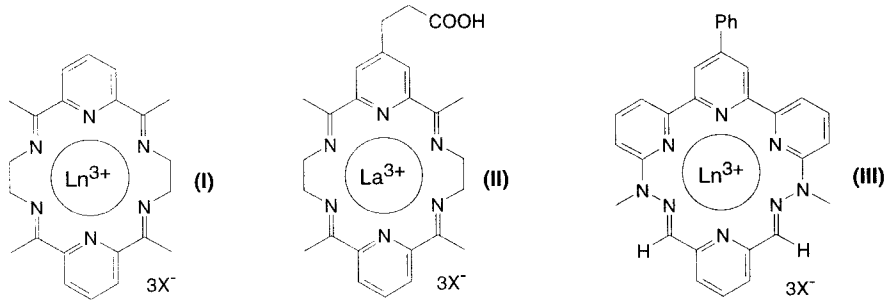


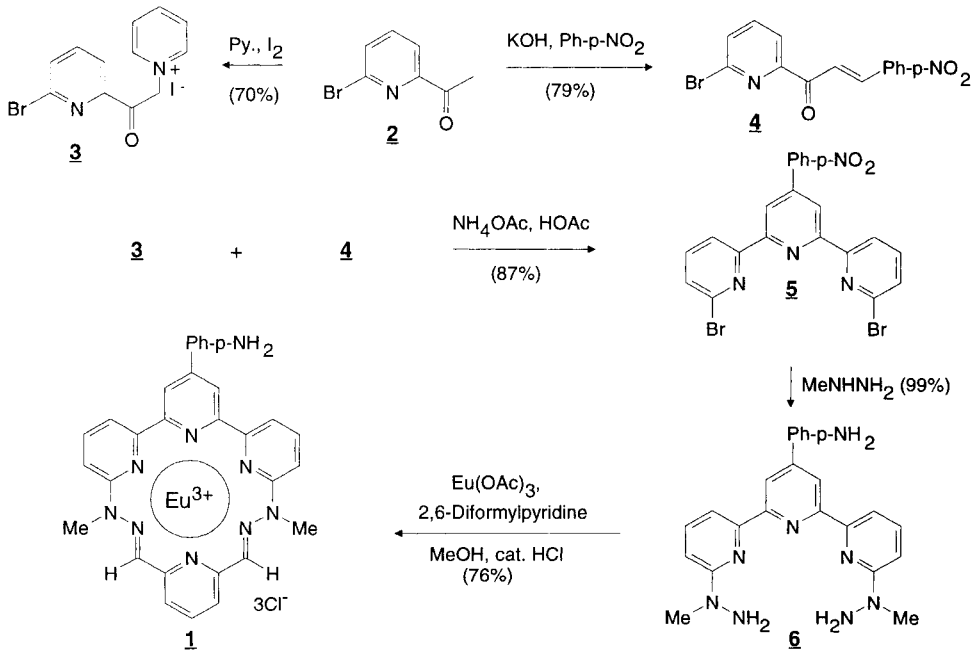
FIG. 1

Despite decomposition of this metal complex on the conjugate by hydrolysis of the imines, we were able to demonstrate cleavage of a complementary strand of synthetic RNA (unpublished results).

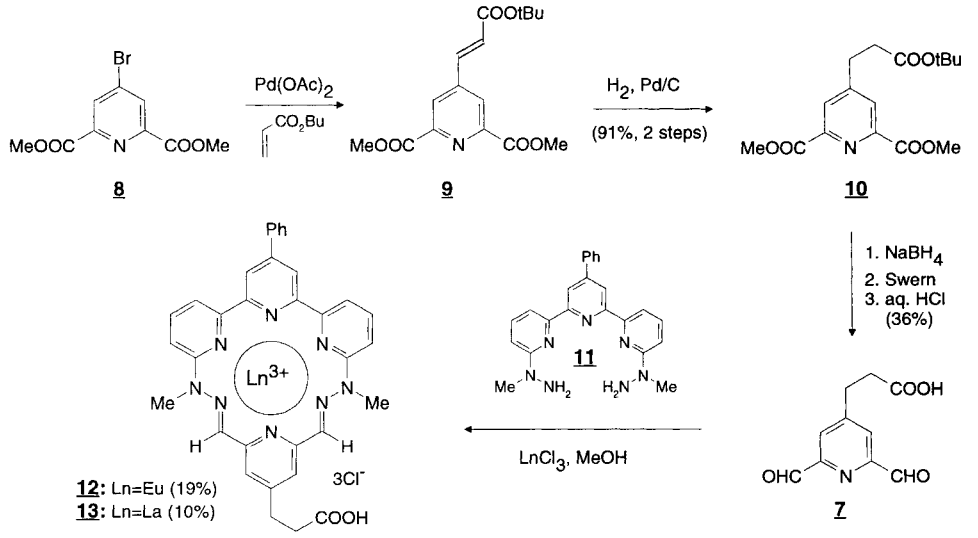
Macrocyclic metal complexes (**III**), described first by Constable,[17] provided a solution to the problem of decomposition with replacement of the ethylenediamine units by pyridylhydrazone groups. These are more resistant to hydrolysis, and both the kinetic inertness of the lanthanide complexes and the ability to cleave RNA are retained. The introduction of an amino group into the terpyridine, so as to allow conjugation of the complex **1** to an amino-oligonucleotide, was carried out with minor modification to the reported synthetic procedures[17] *via* intermediates **2**-**6** (SCHEME 1).

An alternative route for conjugation, was provided by use of a carboxyethyl pyridine dialdehyde **7**, prepared in four steps from chelidamic acid *via* intermediates **8**[18]-**10**. Condensation of **7** with terpyridine **11** and a lanthanide salt gave complexes **12** and **13** (SCHEME 2). Having available the means to conjugate an oligonucleotide to both halves of the macrocycle provided a large degree of flexibility for subsequent structural modification of the complexes and conjugation to oligonucleotides.

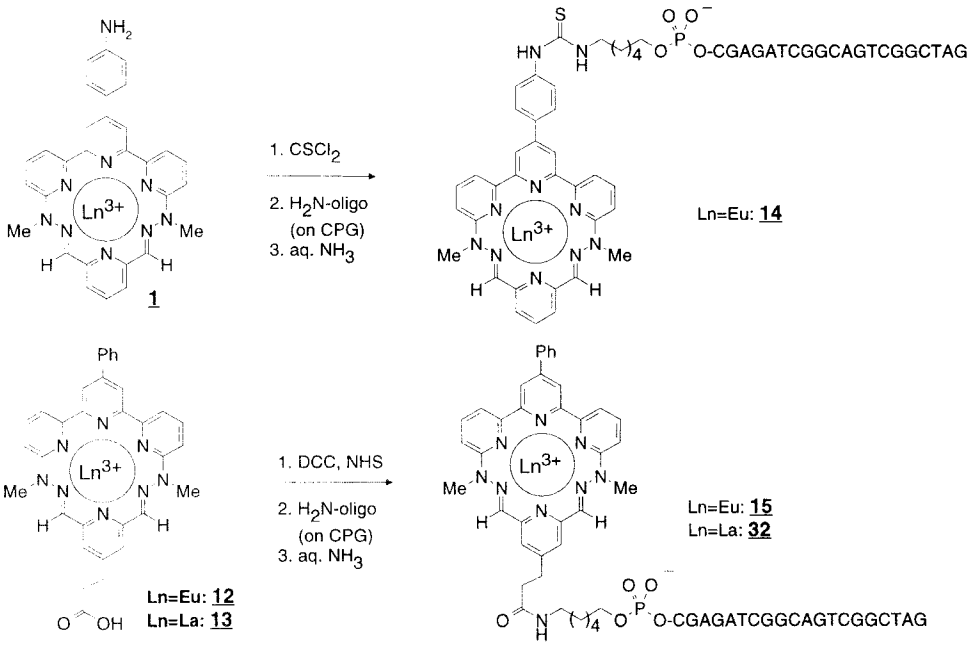
Covalent attachment of complexes **1**, **12**, and **13** to oligonucleotides was carried out as shown in SCHEME 3. Having established that sequence specific cleavage of complementary RNA was possible using these types of oligonucleotide Eu-conjugates,[9] we turned our attention to the rate of the cleavage reactions. One of the requirements for a cleaver conjugate to be useful as an antisense agent is a rapid cleavage reaction.[19] The Eu-derivatives of the first generation conjugates (SCHEME 3; **14**, **15**) cleaved a synthetic RNA with $T_{1/2}$ = 10 h when used in excess over the RNA substrate at 37°C.[10] It was desireable to shorten this $T_{1/2}$ without compromising the metal complex stability nor the



SCHEME 1



SCHEME 2



SCHEME 3

sequence specificity of the conjugates in the cleavage reaction.[19] It seemed reasonable to assume that this could be achieved with modifications to the structure of the complex.

The parent complex has a number of ancillary groups (FIG. 2; Ar, R, Ln, n) that allow a systematic modification of the structure. As knowledge about the mechanism by which these complexes cleave their RNA targets is limited, as wide a range as possible of structural changes were investigated. To help ensure that the kinetic inertness and chemical stability of new derivatives were retained, the terpyridyl part of the molecule was not modified.

Examination of the use of a shorter spacer group (FIG. 2: n=1) between the complex and the oligonucleotide required little synthetic effort; the commercially-available C-3 amino-linker was coupled to the 5'-end of the DNA in place of the C-6 (n=4) unit used in the reference compounds (see TABLE 1).

Synthesis of the complex **16** lacking the 4'-Ph substituent was effected from known compounds **17** and **18**[20] (SCHEME 4). If the Ph-group of the reference conjugate **15** caused some disfavourable steric interactions with the RNA during the transesterification reaction, a gain in cleavage potency could be reasonably anticipated if this group were to be removed.

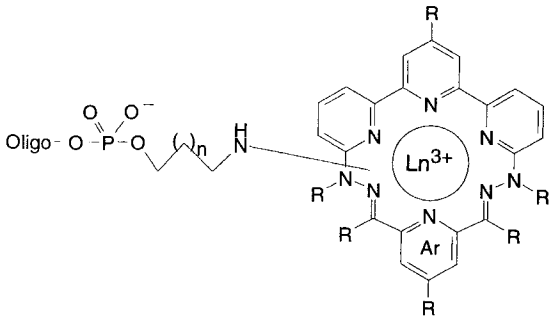


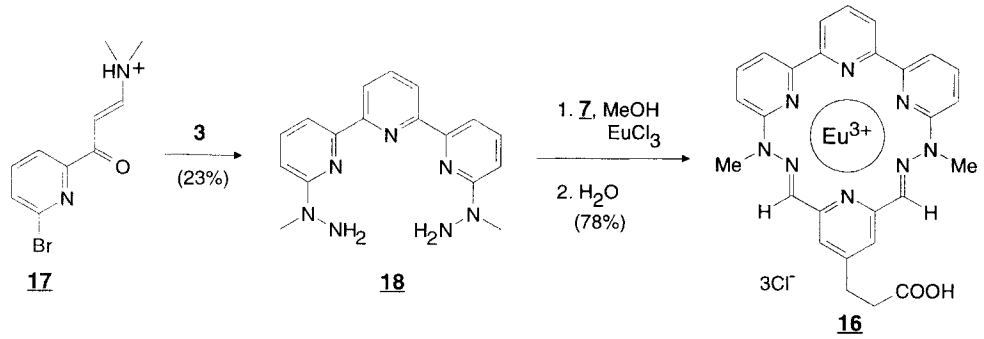
FIG. 2

TABLE 1: mass spectral data of conjugates and results from cleavage experiments using RNA **40**, illustrated in FIG. 3. Conjugates **14**, **15**, **32**, **34**-**39** were synthesized from amino-oligonucleotide **31** (n=4), conjugate **33** was prepared from **31** (n=1).

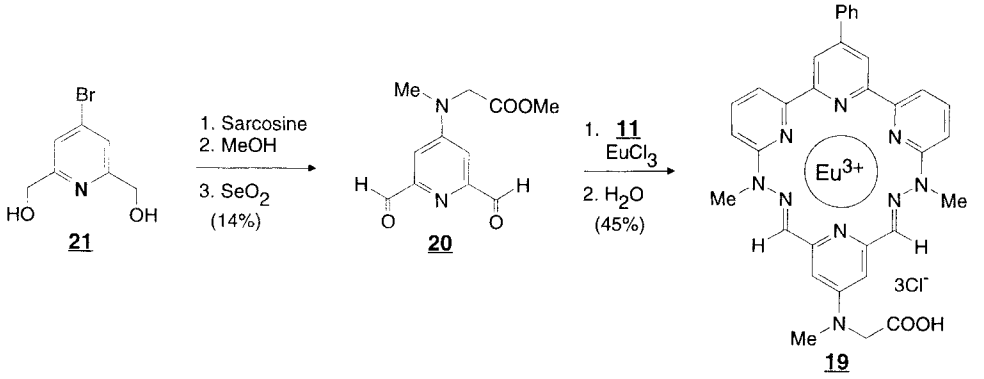
Complex (SCHEME)	L	Conjugate	Mass calc.	Mass found	Lanes (100, 500 nM)	Cleavage yields (%) (100, 500 nM)
1 (1)	thiourea	**14**	7064	7066	10, 11	16, 60
12 (2)	amide	**15**	7061	7065	20, 21	38, 72
13 (2)	amide	**32**	7048	7072	22, 23	1, 8
12 (2)	amide	**33**	7019	7021	12, 13	22, 82
16 (4)	amide	**34**	6984	6991	16, 17	22, 84
19 (5)	amide	**35**	7076	7097	14, 15	13, 64
22 (6)	amide	**36**	7095	7090	18, 19	8, 47
28 (7)	thiourea	**37**	7173	7178	6, 7	2, 17
29 (7)	thiourea	**38**	7098	7090	8, 9	11, 38
30 (8)	thiourea	**39**	7174	7177	4, 5	7, 26

Introduction of a 4-methylamino group on the pyridyl unit of the complex (**19**) was effected from the requisite pyridine dialdehyde **20**, synthesized from known diol **21**[18] (SCHEME 5).

The consequences of placing a strong electrodonating group on the periphery of the macrocycle without changing significantly the steric requirement of the metal complex, were of interest because increasing increasing charge on the metal centre would lower the



SCHEME 4

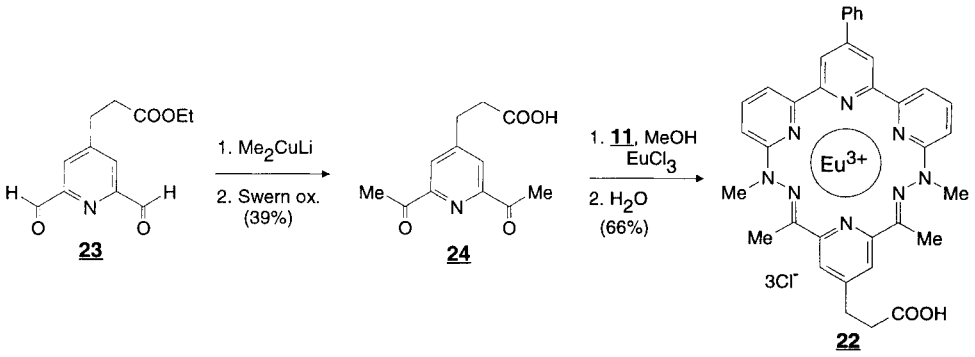


SCHEME 5

Lewis acidity of the metal complex and thus probably decrease the level of RNA cleavage activity. Conversely however, in the unlikely event that this amino group would be protonated in aqueous solution (strong electronwithdrawing imine substituents and a +3 charged metal centre reduce this likelihood), then a higher cleavage activity might be observed.

The effect of alkyl substituents at the imine groups was investigated with complex **22**. Thus, methyl groups were added to the parent complex **12** *via* **23** and **24** in a four step process (SCHEME 6).

Replacement of the pyridyl group of the parent complex **1** with a 5-ring aromatic unit was expected to give metal complexes with altered RNA cleavage activity; whether this would be higher or lower than that of the parent compounds was not obvious. Both 2,4-disubstituted esters of imidazoles,[21] and 2,4-functionalized triazole diols[22] are known.



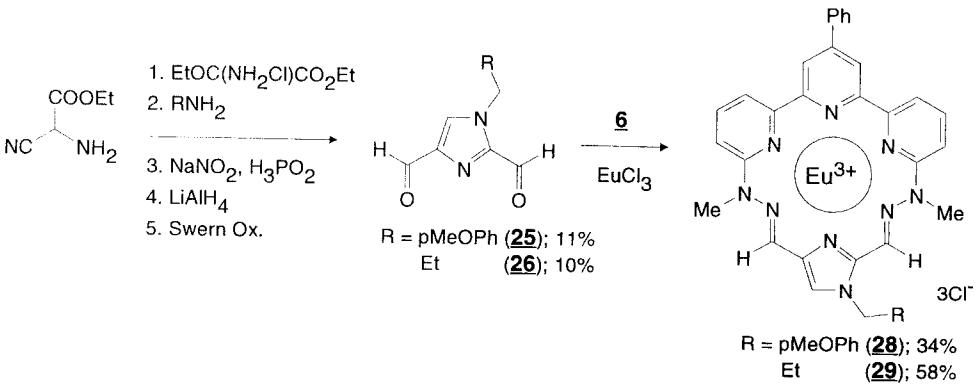
SCHEME 6

Thus, diformyl derivatives of imidazole (**25**, **26**) and triazole (**27**) were prepared (SCHEMES 7 and 8, respectively). Template reaction of these three derivatives with $EuCl_3$ and ligand **6** under the usual conditions was considerably slower than with the 6-ring analogues, presumably due to additional ring strain. Products (**28**-**30**) were isolated as solids, soluble in DMSO.

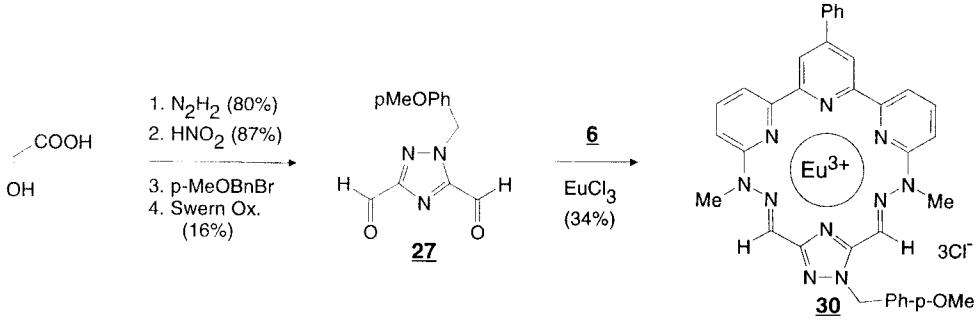
Covalent attachment of new macrocyclic lanthanide complexes to DNA sequences *via* either thiourea or amide linkages was carried out under the standard conditions. Thus compounds **12**, **13**, **16**, **19**, and **22** were activated for conjugation to amino-oligodeoxynucleotide **31** (n=4, n=1 (**12** only)) as their NHS-esters and compounds **28**-**30** were reacted as isothiocyanates (SCHEME 9). Products **14**, **15**, **32**-**39** were purified by reverse-phase HPLC and were characterized by MALDI-TOF mass spectroscopy (TABLE I). The conjugates were tested for cleavage of complementary ribonucleotide **40**. Assays were carried out as described previously[9,11] at two concentrations of the conjugate (100 nM and 500 nM). Quantification of remaining starting material of synthetic RNA (**35**) after 16 h at 37°C using a phosphorimager was used to calculate the yield of cleavage. The results of the experiments are shown in FIG. 3.

Cleavage occurred at the 3'-end of the RNA, adjacent to the cleaver complex. As described previously,[10] reaction appears to occur most readily between 5'-r(G-A) nucleotides; all conjugates gave a similar cleavage pattern, with reaction predominantly between the seventh and eighth residues from the end of the duplex region. Higher cleavage yields were observed at the higher concentration (500 nM) of conjugate.

The two parent conjugates **14** and **15** gave cleavage of the complementary RNA target in yields of 60% and 72% respectively (lanes 11 and 21).



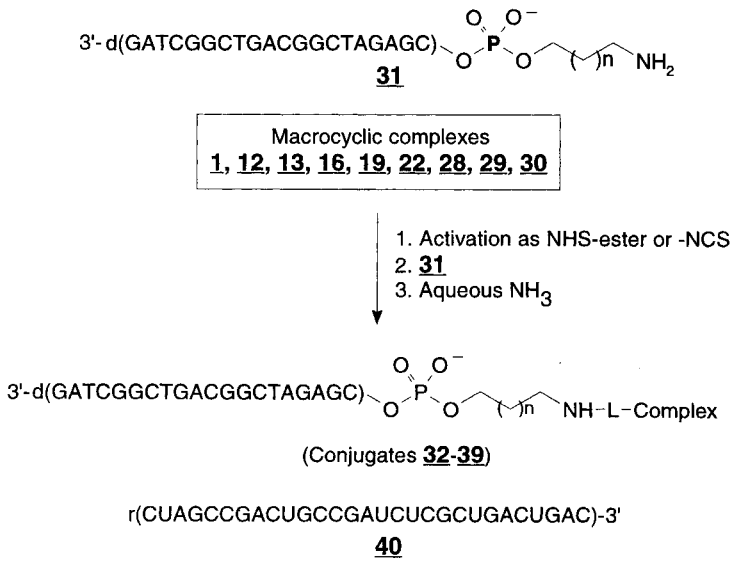
SCHEME 7



SCHEME 8

All three of the 5-ring analogs **37**-**39** showed a lower cleavage yield of the target (lanes 4-9) compared to **14**. Reasons for this are not yet clear. It seems unlikely that it is due to differences between the steric demands of pyridyl and triazolyl/imidazolyl groups as such. It may however reflect a differing topography of the two classes of metal complex leading to differences in coordination of non-covalently bound ligands e.g. H_2O, phosphodiester. Alternatively, weaker Lewis acidity of these complexes or altered acidities of Eu-bound water molecules can also account for the differences in potency.

The use of a shorter linker group (from **31**, n=1) between the complex and the oligonucleotide (conjugate **33**) showed increased cleavage efficiency at 500 nM (lane 13; 82%) compared to the parent conjugate **15** (72%), but a decrease in activity at 100 nM (22% against 38%; lanes 12 and 20, respectively). The cleavage patterns from the two



SCHEME 9

conjugates are identical. This result is not considered to be significant as the structural change $(CH_2)_6 \rightarrow (CH_2)_3$ is small when both the flexibility of these large macromolecules and the overall distance between the metal centre and the last base of the DNA sequence are considered.

Removal of the phenyl substituent from the complex (compound **16**) yielded the most active cleaver conjugate (**34**) at 500 nM, with 84% of the RNA target destroyed (lane 17). However, again at the lower concentration, the reference compound **15** was better (38% compared to 22%; lanes 20 and 16, respectively).

Introduction of the *para*-amino substituent on the solitary pyridine of the macrocycle (conjugate **35**) led to a slight loss in activity compared to **15** (64% and 72%; lanes 15 and 21, respectively). This result, however, is less dramatic than that resulting from substitution of methyl groups for hydrogen (conjugate **36** from complex **22**) at the imines of the parent complex **12** (47% against 72%; lanes 19 and 21, respectively). This surprising effect has also been confirmed with the dimethyl analogue of **14** (results not shown) and observed with a variety of conjugates targeted to different RNAs. The origin of the effect is unknown. It might be steric in nature; groups at this position are relatively close to the metal centre and might interfere with binding of the reacting phosphodiester. Alternatively, a steric interaction with the methyl substituents of the hydrazino groups

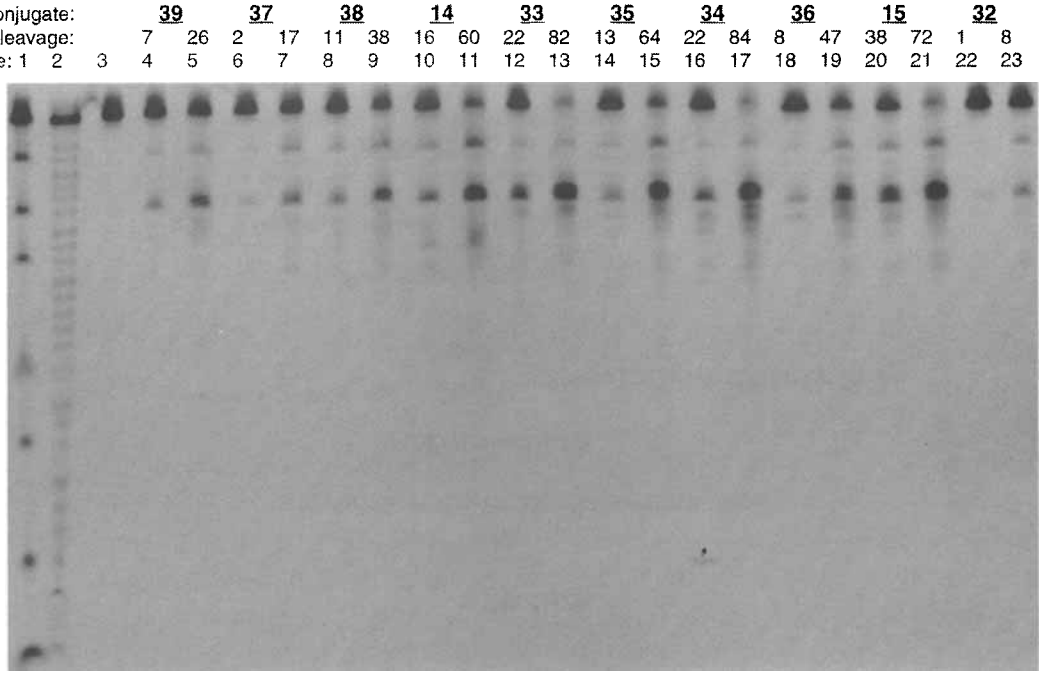


FIG. 3: Autoradiograph of a 12% denaturing polyacrylamide gel after treatment of ^{33}P-labelled RNA **40** (16h, 37°C, pH 7.4) with conjugates (see TABLE 1). Lanes 1,2; treatment with RNase T_1, Base respectively; lane 3: **40** alone. Lanes 4, 6, 8, 10, 12, 14, 16, 18, 20, 22: 100 nM conjugate; lanes 5, 7, 9, 11, 13, 15, 17, 19, 21, 23: 500 nM conjugate.

might induce changes in the topography of the complex, e.g. buckling, thus altering coordination of anions and hence cleavage efficiency. Efforts to obtain crystal structures of these complexes are underway.

CONCLUSIONS: A variety of structural changes were made to the two parent complexes **1** and **12** with the goal of increasing cleavage activity of their respective oligonucleotide conjugates. These changes were evaluated in a series of experiments that compared head-to-head cleavage potency of the new derivatives with the parent compounds against a complementary synthetic RNA.

Although several different complexes were synthesized, conjugated and tested, none of these provided as large a jump in activity as that observed in passing from La- to Eu-derivatives (e.g. from **32** to **15**). At 500 nM concentration of conjugate, **34**, **33** and the reference conjugate **15** were the most active compounds. At the lower concentration (100 nM), **15** was the most potent of the series. The results are summarized as i) the Eu-

derivatives give the most active complexes; ii) H is the preferred substituent at the imino-carbon; iii) 6-ring pyridyl substituent is preferred to 5-membered aromatic rings and; iv) conjugation *via* the pyridyl ring generally gives more active conjugates.

Conjugation of these complexes to nuclease-stable oligonucleotides and testing of the compounds in cellular assays is underway.

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