Efficient sequence-specific cleavage of RNA using novel europium complexes conjugated to oligonucleotides

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Background: A general method allowing the selective destruction of targeted mRNA molecules *in vivo* would have broad application in biology and medicine. Metal complexes are among the best synthetic catalysts for the cleavage of RNA, and covalent attachment of suitable metal complexes to oligonucleotides allows the cleavage of complementary single-stranded RNAs in a sequence-specific manner.

Results: Using novel europium complexes covalently linked to an oligodeoxyribonucleotide, we have achieved the sequence-specific cleavage of a complementary synthetic RNA. The complexes are completely resistant to chemical degradation under the experimental conditions. The cleavage efficiency of the conjugate strongly depends on the nature of the linker between the oligonucleotide and the complex. Almost complete cleavage of the RNA target has been achieved within 16 h at 37 °C.

Conclusions: The results will be important for improving the efficacy of antisense oligonucleotides and will provide a basis for the design of synthetic RNA restriction enzymes. Conjugates of the kind described here may also find application as chemical probes for structural and functional studies of RNA.

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Introduction

The sequence-specific hydrolytic cleavage of ribonucleic acid (RNA) by a complementary oligonucleotide conjugated to a chemical cleaving moiety has long been an important goal of chemists [1,2]. Besides providing useful tools in molecular biology [3,4], such conjugates could have important consequences for the antisense field [5–7]. It is becoming increasingly clear that most antisense applications will require the selective destruction of a targeted mRNA; in most cases the only derivatives of antisense oligonucleotides which give potent biological activity are those that can induce enzymatic degradation of RNA [8–11]. Therefore, a general method for cleaving targeted RNA sequences is desirable.

For an artificial ribonuclease to be of practical use in a therapeutic sense, several requirements have to be satisfied. First, it must give efficient cleavage of the target RNA under physiological conditions. Second, it must be stable *in vivo*. Third, ideally, it should operate with catalytic turnover and without the need for exogenous cofactors. Last, it should be readily synthesized. There are few known cleavage functionalities that meet all of the above criteria. Some metal complexes are reported to cleave RNA by a hydrolytic pathway [12–23] but it is often particularly difficult to construct complexesof any kind so as to be stable *in vivo* [24,25]. Recently, the sequence-specific hydrolytic cleavage of RNA by metal complexes conjugated to oligonucleotides has been achieved. Bashkin and coworkers reported scission of a run-off transcript using a terpyridyl-Cu(II) complex conjugate (18–25 % target cleavage, 45 °C, 72 h) [21]. Shortly after this, Komiyama and coworkers described the cleavage of a synthetic RNA using an iminodiacetate-Lu(III) conjugate (17 % cleavage, 37 °C, 8 h) [23]. Both of these approaches employed metal complexes formed *in situ* by adding metal salt to a solution of the DNA–ligand conjugate. The noteworthy contribution from Magda and coworkers [22] described the conjugation of a stable preformed Eu(III) complex to DNA to give a reagent which reached ~30 % cleavage of its target under similar conditions (37 °C, 18–24 h). Here we describe the covalent attachment of novel stable europium complexes to oligodeoxynucleotides and the near-quantitative cleavage of a complementary strand of synthetic RNA in 16 h at physiological pH and temperature.

Results and discussion

The ability of rare earth metal salts to accelerate phosphodiester hydrolysis is well-documented [17, 26–29]. Macromonocyclic [12–14] and bicyclic [30] lanthanide complexes are known to be efficient promoters of RNA cleavage and efforts to identify suitable complexes for this purpose led us to macrocyclic compounds of type A (Fig. 1). These complexes are structurally similar to compounds of type B, which were first reported by Morrow *et al.* [12] to cleave RNA by a *trans*-esterification pathway. However, as the authors also reported that these complexes undergo slow decomposition [12], they were considered unsuitable for our needs. Replacement of the imine by hydrazone-type linkages, on the other

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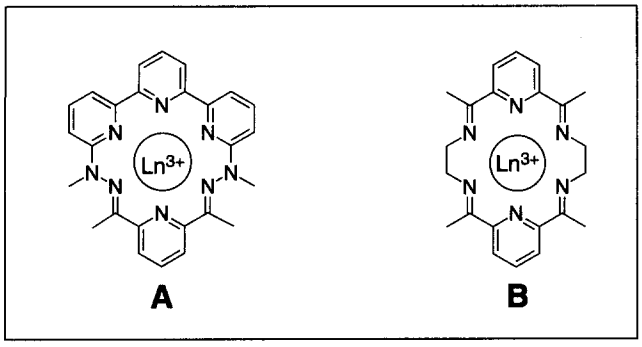

Fig. 1. Eighteen-membered macrocyclic lanthanide complexes. Compounds of type A are used in this study; type B were previously employed by Morrow *et al.* in RNA cleavage reactions [12]. Ln, lanthanide.

hand, provided complete protection against hydrolytic decomposition of the complex in aqueous solution. Thus, the terpyridine-derived europium complexes, shown as compounds **1–3** in Fig. 2, were prepared in analogy to the corresponding nickel(II) complex [31].

Complexes **2** and **3** were linked to the 5'-hexylamino-derivatized oligonucleotide **4** (Fig. 2) via the corresponding isothiocyanate and N-hydroxysuccinimide ester derivatives, respectively. Standard HPLC purification afforded the pure conjugates **5** and **6**, which were fully characterized by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), capillary gel electrophoresis (CGE), UV spectroscopy and polyacrylamide gel electrophoresis (PAGE) analysis. The stability of compound **6** was tested by incubating it at 37 °C for nine days in each of the following buffers: 20 mM Tris buffer (pH 7.7), 2 mM acetate buffer (pH 4.7) and 5 mM EDTA solution (pH 8.0) [32]. No decomposition was detectable by PAGE.

To test whether complexes **5** and **6** could selectively cleave RNA, the synthesized 29-mer oligoribonucleotide **7** (Fig. 2), which contains a sequence at the 5' end that is complementary to the sequence of compound **4**, was used as the target. Products were analyzed on a 12 % denaturing polyacrylamide gel (Fig. 3). Incubation of the target RNA with europium (III) acetate (400 μM, lane 6) or the complexes **1** and **3** (≥40 μM, lanes 8–10 and 12–14) led to cleavage of the whole target strand. Lower concentrations of these complexes (1 μM, lanes 11 and 15) did not cleave the RNA. On the other hand, treatment of the target **7** with the complementary 20-mer oligodeoxynucleotide conjugates **5** and **6** (400 nM, lanes 16 and 20) resulted in scission of the target strand exclusively in the approximate region where the metal complex is expected to be located (Fig. 4). Hard Lewis

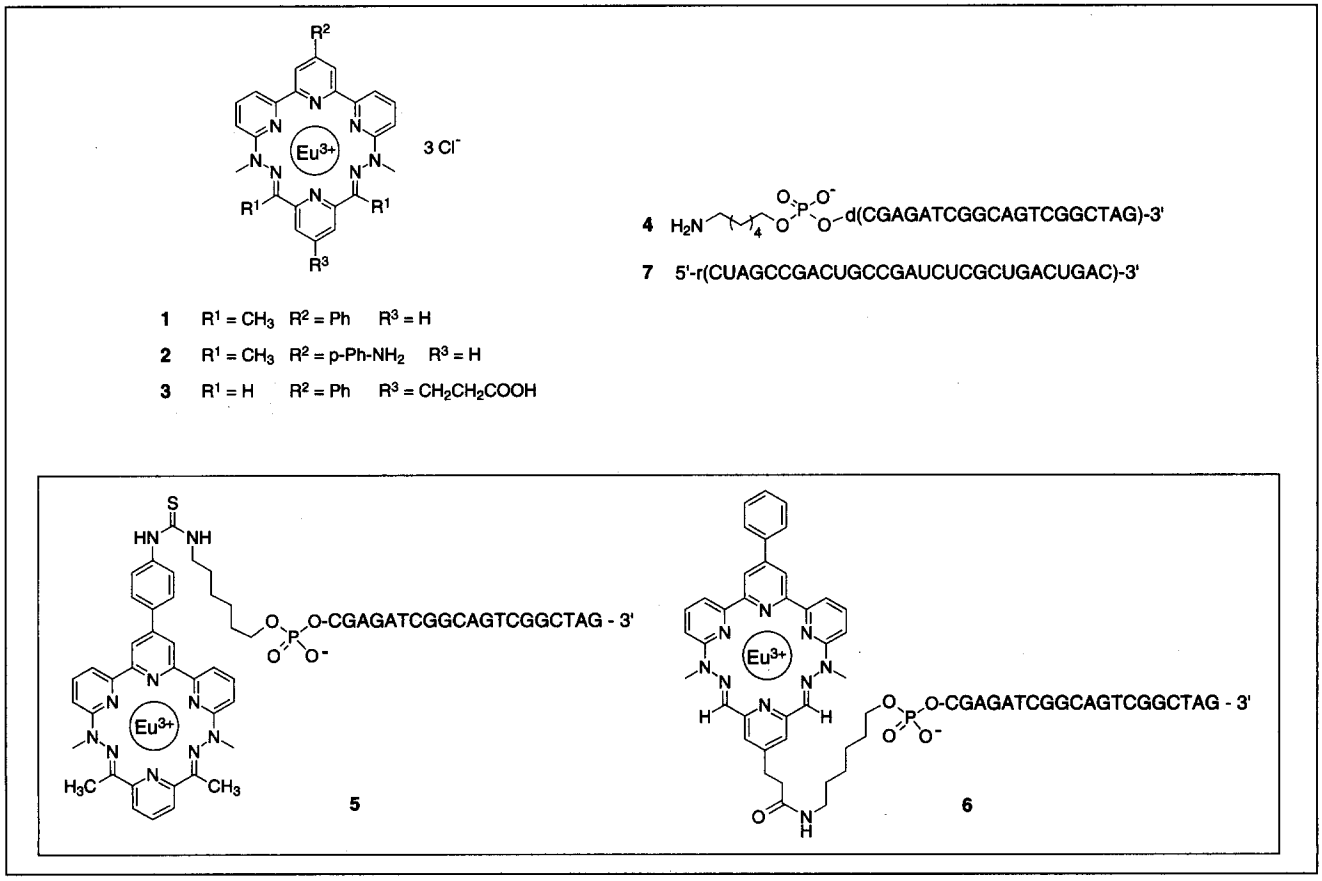

Fig. 2. Structure of terpyridine-derived europium complexes linked to a DNA strand. The targeted conjugates **5** and **6** were obtained by covalent attachment of complexes **2** and **3** to the amino-oligodeoxynucleotide **4**. The sequence of **4** is complementary to the 5'-end of oligoribonucleotide **7**, which is used as the target in cleavage assays.

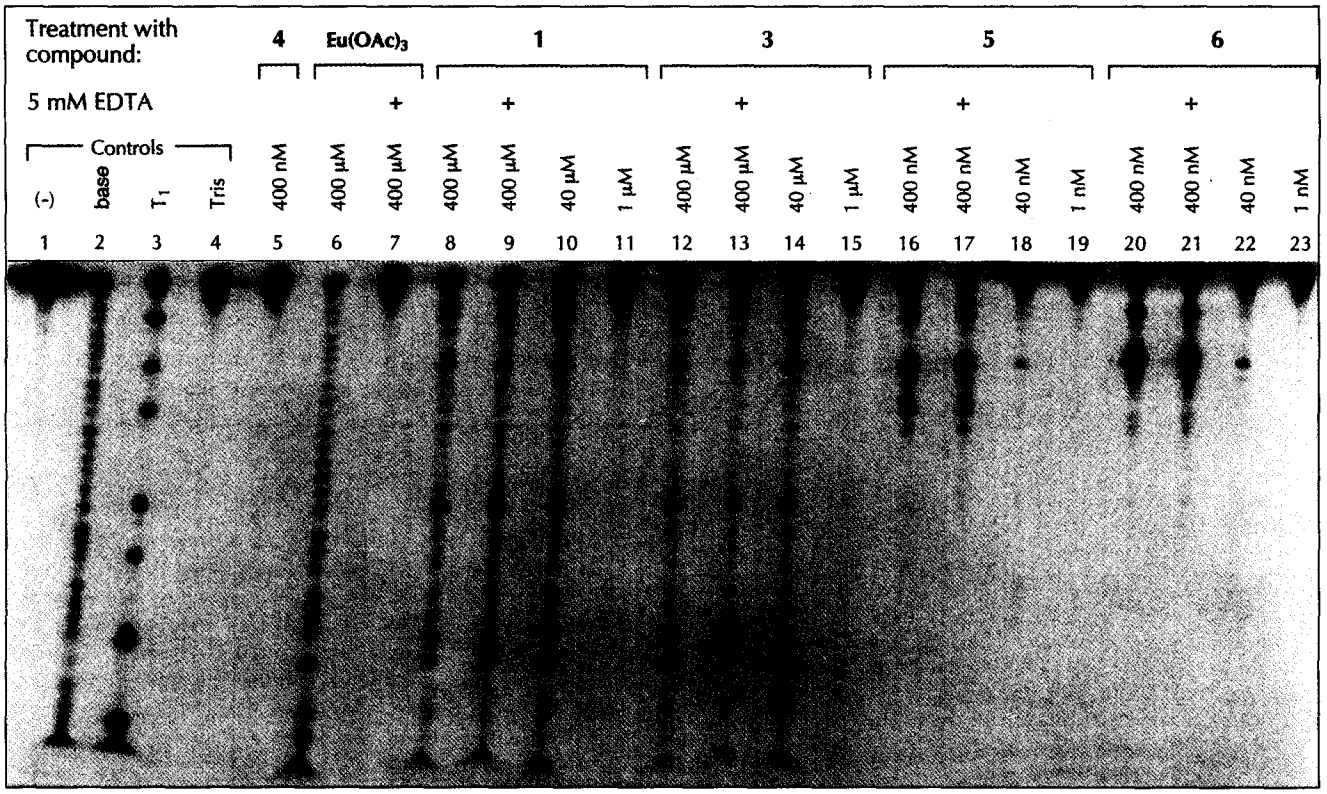

Fig. 3. Autoradiograph of a 12 % denaturing polyacrylamide gel obtained after treatment of the 29-mer oligoribonucleotide **7** (^{33}P labelled at the 5'-end) with free metal complexes **1** and **3** and conjugates **5** and **6**. Lanes 1 and 4: RNA before and after incubation in the reaction buffer; lane 2: alkaline hydrolysis (40 mM NaOH, 70 °C, 20 min); lane 3: RNase T1 digestion; lanes 5–23: treatment with the compounds indicated (16 h, pH 7.5, 37 °C).

acids are known to bind strongly to phosphate groups of nucleic acids [33]. A plausible mechanism therefore involves coordination of phosphate to europium facilitating *trans*-esterification and thus backbone cleavage by intramolecular attack of the 2'-hydroxyl group. Other factors, such as deprotonation of the 2'-hydroxyl group by a metal-bound hydroxide or stabilization of the leaving 5'-hydroxyl group, may also be involved in the metal assisted cleavage process [16,17,30,34–36].

Neither the efficiency nor the pattern of cleavage was affected by the presence of 5 mM EDTA (Fig. 3, lanes 17 and 21). However, EDTA completely inhibited the cleavage seen with the free europium (III) salt, (lane 7). Cleavage assays carried out with **5** and a different RNA target (data not shown) in both Tris and phosphate buffers (20 mM) showed that phosphate does not inhibit the reaction nor affect the efficiency and pattern of cleavage As expected, no cleavage was observed after treatment with amino oligonucleotide **4** alone (lane 5). Incubation with a combination of **4** and the unattached complexes **1** and **3** (≥ 40 µM, data not shown) gave only non-specific cleavage preferentially in the single-stranded region.

After 16 h, conjugate 5 (400 nM, 37 °C) had effected 51 % cleavage of the target strand, primarily at the third residue (a guanosine) from the end of the duplex (Fig. 5a). The site selectivity observed in this particular case may originate from a natural hot-spot in the target strand, one of several revealed by cleavage using **1** (see Fig. 3, lanes 8–10). Secondary cleavage sites were observed in the single-stranded region, at a more distant guanosine and close to the last residue of the duplex. Treatment of the target oligonucleotide **7** under the same conditions with

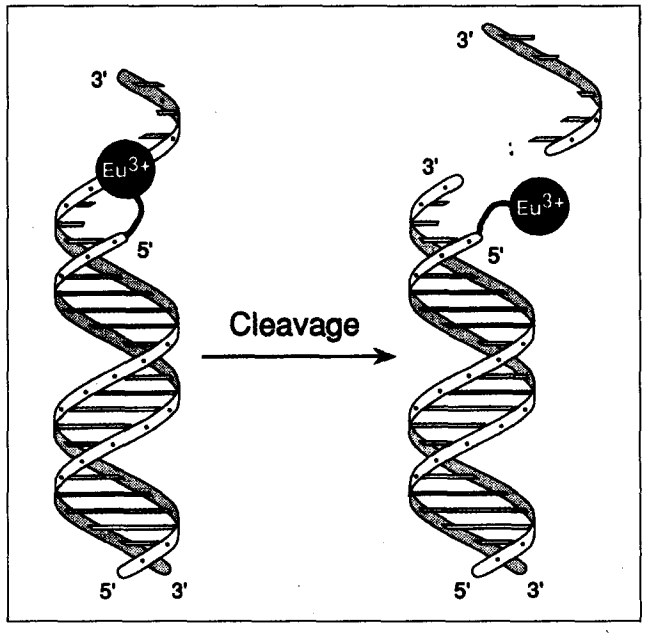

Fig. 4. Cleavage of compound **7** by Eu(III)-complex DNA conjugates is specific to a region close to the metal center.

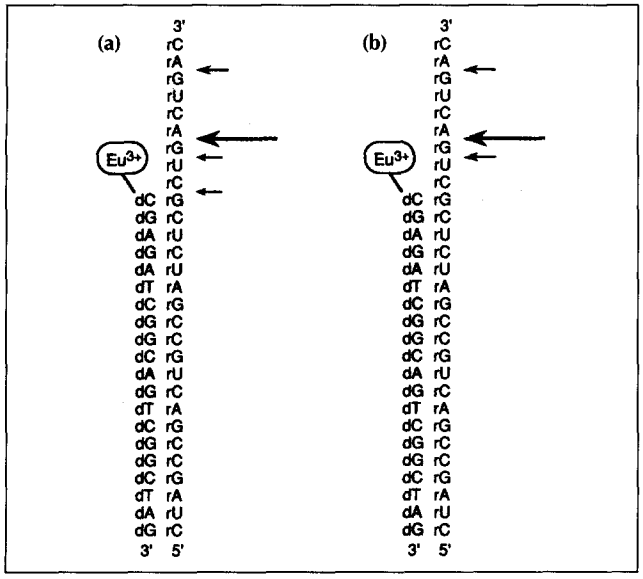

Fig. 5. Cleavage pattern obtained after treatment of compound **7** with Eu(III)-complex DNA conjugates. **(a)** Treatment with conjugate **5**. **(b)** Treatment with conjugate **6**. Large arrows show major sites of cleavage, small arrows minor sites.

conjugate **6** resulted in 88 % cleavage of the RNA target almost exclusively at one site, again the third residue of the single-stranded region. Fig. 5b shows the detailed cleavage pattern. The relatively large difference in the activity between conjugates **5** and **6** is believed to derive from the nature of the linker groups. More active compounds may become available through further modification of the linker with regard to length, rigidity and site of attachment at the metal complex, or alternatively, with changes in the cation and/or the ligand.

Significance

The sequence-specific cleavage of RNA is currently an area of intensive investigation. Besides providing useful tools for molecular biologists, artificial ribonucleases also have the potential of being used as drugs. Antisense oligonucleotides have been shown to inhibit the expression of proteins by selective interference at the RNA level [5–7]. The most common pathway of this inhibition is the RNase H-mediated degradation of the targeted RNA after formation of a hybrid duplex with an antisense oligodeoxynucleotide [9–11]. However, chemical modification is required to protect the oligonucleotides against nucleolytic degradation resulting — with a few exceptions — in the loss of this natural mechanism of RNA cleavage and therefore a decrease in biological activity. Covalent attachment of the antisense oligonucleotide to a chemical moiety which can cleave RNA could provide a general solution to this problem.

We have described the sequence-specific cleavage of a single-stranded RNA through the use of novel, chemically stable, macrocyclic europium complexes covalently linked to a complementary oligodeoxynucleotide. The cleavage efficiency of the conjugates, which are highly resistant to decomposition under the experimental conditions, shows significant dependence on the type of linkage between the oligonucleotide and the complex. Almost complete cleavage (88 %) of the RNA target at 37 °C within 16 h was achieved upon treatment with a conjugate at 400 nM concentration. The conjugates described provide a solid basis for further development of artificial ribonucleases acting in a catalytic way.

Materials and methods

Preparation of oligonucleotides

Oligonucleotides were prepared by automated synthesis on a 1.5 μmol scale on a DNA synthesizer (Applied Biosystems Inc. 394A-08), using commercially available reagents. DNA and 4-monomethoxy trityl (MMT) amino-C6 cyanoethyl phosphoramidites were obtained from MWG-Biotech GmbH, Expedite™ RNA cyanoethyl phosphoramidites from Millipore. Oligodeoxynucleotide **4** (5'-amino group MMT-protected) was cleaved from the support and base-deprotected by treatment with concentrated aqueous ammonia at 55 °C overnight. After removal of the ammonia the crude material was purified by HPLC using a semi-preparative RP-C18 column [Hypersil™, 5 μm particle size; 50 mM triethylammonium acetate (TEAA, pH 7.0) starting with 10 % acetonitrile increasing with a gradient of 0.7 % min^{-1}]. After concentration of the fractions containing the oligonucleotide, the MMT group was removed by treatment with 80 % aqueous acetic acid for 30 min. The final purity of the oligonucleotide was >95 % as assessed by CGE. MALDI–MS m/z 6363; expected for $[M-H]^-$: 6361.

Oligoribonucleotide **7** was cleaved from the support and base-deprotected by treatment with a mixture of concentrated aqueous ammonia (25 % v/v) in ethanol for 16 h. The 2'-protecting group (*tert*-butyldimethylsilyl) was removed with a 1 M solution of tetrabutyl-ammonium fluoride in tetrahydrofuran for 24 h at room temperature in the dark. The reaction mixture was quenched with an equal volume of 50 mM triethylammonium hydrogencarbonate (TAHC) solution (pH 7.0), dialyzed against 7.5 mM TAHC (pH 7.0) at 4 °C, concentrated and used for 5'-end labelling as described below.

Preparation of conjugate 5

A solution of complex **2** (100 mg, 0.125 mmol) in water (3.5 ml) was added to a mixture of $NaHCO_3$ (46 mg, 0.55 mmol) and thiophosgene (35 μl, 0.44 mmol) in chloroform (3.5 ml). The mixture was vigorously stirred for 2.5 h at room temperature and filtered. The aqueous phase was separated and concentrated. The isolated isothiocyanate was used in the following step without further purification. MALDI–MS m/z 804.7; expected for $[M-Cl]^+$: 804.6.

The crude isothiocyanate (1.0 mg, ~1 μmol) was added to a solution of the amino oligonucleotide **4** (27 nmol) in a mixture of pyridine/water/triethylamine (90:15:1; 150 μl). The reaction mixture was allowed to stand at room temperature for 1 h. It was then dialyzed against 0.1 M aqueous KCl and three times against water. The solution was lyophilized and the pure product (>90 % by CGE) was obtained from reverse phase HPLC

[Hypersil™ 5 μm C18 ODS, 4.6 x 125 mm; flow: 1 ml min^{-1}, starting with 100 % 50 mM TEAA (pH 7.0) and using an acetonitrile gradient of 0.35 % min^{-1}; retention time of compound **5**: 40 min]. UV (λ_{max}) nm 257, 363. MALDI–MS *m/z* 7090; expected for $[M-H]^-$: 7091.

Preparation of conjugate **6**

N-Hydroxysuccinimide (0.4 mg, 3.3 μmol) in DMSO (25 μl) was added to a DMSO solution (200 μl) of compund **3** (2.9 mg, 3 μmol) at room temperature. A DMSO solution (25 μl) of dicyclohexyldiimide (0.6 mg, 3.3 μmol) was injected. The solution was allowed to stand for 16 h. Neat diisopropylethylamine (18 μl, 100 μmol) was added, followed by an aqueous solution (80 μl) of the amino-oligonucleotide **4** (27 nmol). After standing overnight at room temperature, the solution was diluted with water and dialyzed twice against 50 mM TAHC (pH 8.5) for 24 h. Salts were dialyzed out of the mixture and after lyophilization, the pure product (>90 % by CGE) was obtained from reverse phase HPLC (same conditions as above; retention time: 39 min). UV (λ_{max}) nm 256, 334, 379. MALDI–MS *m/z* 7065; expected for $[M-H]^-$: 7060.

RNA 5'-end labelling

Special precautions were taken to prevent contamination by traces of metals and nucleases as described [37]. The oligoribonucleotide **7** (100 pmol) was ^{33}P end-labelled by treatment with 5 units of T4 polynucleotide kinase (Promega), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM spermidine, 50 mM Tris-HCl (pH 7.5) and 5 μCi [γ-^{33}P]ATP (Amersham, 1000 Ci/mmol) in a total reaction volume of 20 μl. The mixture was incubated at 37 °C for 30 min followed by ethanol precipitation. After addition of 15 μl loading buffer (containing each 0.025 % xylene cyanol and bromphenol blue in a 1:1 mixture of 80 % deionized formamide and 7 M urea containing 20 mM citric acid and 1 mM EDTA) the mixture was heated for 5 min at 95 °C, chilled on ice and loaded on a 12 % denaturing polyarylamide gel. After electrophoresis (2.5 h, 55 W) the RNA band was cut out, electroeluted and ethanol-precipitated. The material labelled by this procedure contained 200 000 cpm (Cerenkov protocol).

RNA cleavage assay

The 5'-end-labelled oligoribonucleotide **7** (12 000 cpm, final concentration estimated to 10–50 nM) and the corresponding oligonucleotide conjugate (400 nM, 40 nM or 1 nM final concentrations) or complex (400 μM, 40 μM or 1 μM final concentrations) were dissolved in 50 mM Tris-HCl buffer (pH 7.4; total reaction volume of 10 μl). The sample was heated for 1 min to 85 °C, cooled to 37 °C and kept at this temperature for 16 h. The reaction mixtures were diluted with 5 μl loading buffer, heated to 95 °C for 1 min, chilled on ice and loaded on a 12 % denaturing Long Ranger™ gel (AT Biochem). Electrophoretic separation (1.25 h, 60 W) was followed by exposure to X-ray film (Kodak, X-OMAT™ AR) and Phosphorimager™.

Supplementary material available

Characterization of compounds **1–3**.

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