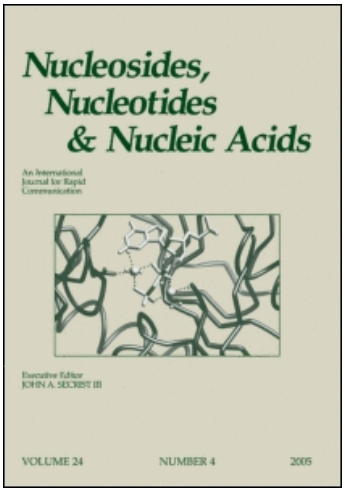

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A METHOD FOR SYNTHESIS OF AN ARTIFICIAL RIBONUCLEASE

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A METHOD FOR SYNTHESIS OF AN ARTIFICIAL RIBONUCLEASE

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ABSTRACT

5-Amino-2,9-dimethyl-1,10-phenanthroline-oligonucleotide conjugates have been synthesized. A 2′-*O*-methyl octaribonucleotide carrying a 2′-aminoethoxymethyl linker in a central position was produced. Reaction of the aminoneocuproine phenyl carbamate with the fully deprotected oligonucleotide in aqueous solution gave virtually quantitative conversion into the conjugate. Preliminary cleavage studies in presence of zinc ions show nuclease activity towards RNA targets.

Oligonucleotide based artificial nucleases for hydrolytic cleavage of RNA have potential for use in the next generation of antisense reagents. Such artificial nucleases are generally designed to cleave target sequences using a tethered catalytic group (1). These compounds have the advantage that catalytic turnover can be achieved without the need of cellular enzymes, like RNase H. The catalytic group can be an acid-base catalyst or a metal chelate. In the present study conjugates of 5-amino-2,9-dimethyl-1,10-phenanthroline and a modified oligonucleotide have been synthesized and evaluated for use as artificial ribonucleases in the presence of Zn^{2+}. Use of the same phenanthroline derivative in a different oligonucleotide based artificial nuclease construct has recently been reported (2). The initial design is based on a 2′-*O*-methyl modified 8-mer (5′-GAGUACUC-3′) oligonucleotide (**1**) carrying a 2′-aminoethoxymethyl linker in a central position (Fig. 1a). The amino linker is used to attach 5-amino-2,9-dimethyl-1,10-phenanthroline via a urea linkage (Fig. 1b).

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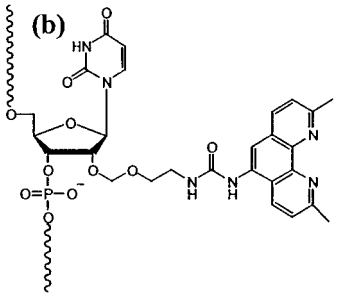
(a) Oligonucleotide Sequences

(1) 5'-mGmAmGUxmAmCmCG-3'
(2) 5'-CGG UAC UC-3'
(3) 5'-CGG UAA CUC-3'

(b)



(c)

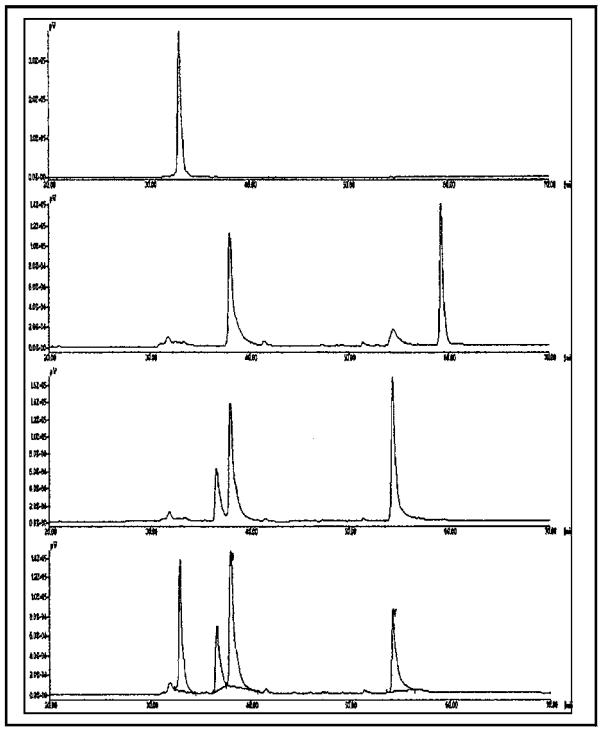


Figure 1. **b** shows a schematic presentation of the conjugate part of **1**. **a** shows the oligonucleotides used in this study, **2** and **3** are substrates for the nuclease. **c** shows HPLC analysis of the conjugation reaction: The top cromatogram is non conjugated. The second is reaction mixture without oligomer. The third spectra shows the crude reaction mixture after incubation for 2 hours. The bottom chromatogram is from mixing the above reaction mixture with non conjugated oligomer. The first peak in the third chromatogram was isolated and identified as the peak corresponding to **1**.

A synthetic route for the preparation of the nucleases has successfully been developed. The catalytic group could be conjugated in aqueous media to the already assembled and deprotected oligonucleotides in a virtually quantitative yield by reacting the free amino group of the linker with the phenyl carbamate of the amino phenanthroline derivative. Neocuproine (**4**) was first converted into the 5-NH_2-analogue (Scheme 1). 5-NO_2-2,9-dimethyl-1,10-phenanthroline (**5**) was

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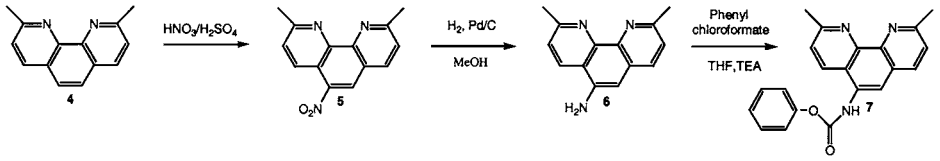
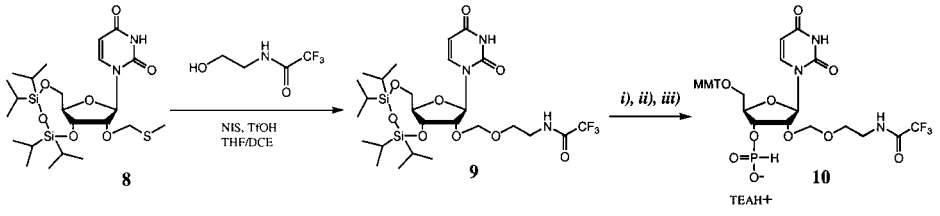


Scheme 1. Synthetic route to the neocuproine reagent.

synthesized from **4** using SO_3/H_2SO_4, HNO_3, 168°C (3). The nitro derivative **5** was then reduced to 5-amino-2,9-dimethyl 1,10 phenanthroline (**6**) by catalytic hydrogenation on palladium/carbon. The amino derivative **6** was then subsequently converted into the phenyl carbamate **7** by reaction with phenyl chloroformate in THF in the presence of triethyl amine (Scheme 1).

The building block bearing the linker arm was synthesized from uridine (Scheme 2) via a previously developed route (Katcevica D., Rozners, E., Bizdena, E. and Strömberg, R., unpublished results). Uridine was first protected with the 1,1,3,3- tetraisopropyldisiloxyanylidene (TIPDS) group. Reaction of the 3′,5′-TIPDS-uridine with DMSO/acetic anhydride/acetic acid (4) gave after silica gel chromatography and crystallization from hexane-ether the desired product **8**. Coupling of **8** with N-protected aminoethanol (made from reaction of aminoethanol with *S*-Ethyl trifluorothioacetate) using NIS/TfOH (5) activation gave the desired product **9**. The silyl protection was removed using fluoride ion and the deprotected compound was converted into building block **10** suited for use in the H-phosphonate method for oligonucleotide synthesis (6). This was achieved by standard monomethoxytritylation in pyridine followed by phosphonylation with the PCl_3/imidazole reagent (7). The oligonucleotides were then made by machine assisted solid phase synthesis using a standard protocol.

The conjugation reaction was performed in sodium tetraborate buffer (pH 8.5) and seemed to be quantitative as judged from analysis by reversed phase HPLC (Fig. 1c). The oligonucleotide conjugate was purified by reversed phase HPLC giving homogeneous products and mass spectrometry analysis confirmed the identity of the conjugate.



i) $Bu_4N^+ F^-$/THF ; *ii)* MMTCl /Pyridine ; *iii)* (a) PCl_3/imidazole/Et_3N (b) water

Scheme 2. Coupling of protectd aminoethanol to **5**.

The thermal stability of the 2′-*O*-Me modified oligonucleotide **1** was determined by means of thermal melting of complex with complementary sequence **2**. In order to determine the effect of the catalytic group on the duplex-stability, the melting temperature was checked both before and after conjugation with **7**. The melting point was determined to 44°C and 39°C respectively.

Kinetic cleavage studies have been initiated using the 5-aminoneocuproine-**1** nuclease to cleave complementary oligonucleotide substrate **2** and sequence **3** that contains an additional adenosine unit in the middle of the sequence. In both cases cleavage was detected in presence of 10-mM Zn^{2+} ions at pH 7. In the case of sequence **3** the cleavage appears to be more sequence selective. In the case of 5-aminoneocuproine-**1** nuclease cleaving substrate **2** the estimated first order rate constant is $2 \times 10^{-4}\,\mathrm{min}^{-1}$.

EXPERIMENTAL

Synthesis of the 5*-aminoneocuproine-***1** *conjugate*. Carbamate **7** (250 μg, 0.73 μmol) was dissolved in 14 μl dry DMSO. To this solution was added: 7 μl H_2O, 75 μl Sodium tetraborate buffer (0.1-M, pH 8.5) and finally a 4 μl (37 nmol) solution of oligonucleotide **1**. The vial containing the reaction mixture was agitated, 2 μl aliquotes were withdrawn from the reaction mixture, filtrated, diluted to 100 μl water and analysed with reversed phase (RP) HPLC. The reaction was incubated overnight although it appeared complete in 2 h. The reaction mixture was then filtered and purified on RP HPLC. 18.3 nmol product corresponding to a yield of 50% was isolated. Both analytical and preparative HPLC was performed on a Hypersil ODS column (5 μm, 4.6 × 25 mm) using 5 min isocratic elution with 0.1-M triethylammonium acetate (*aq*.), then a linear gradient to the same buffer containing 20% MeCN in 35 min and finally from 20 to 50% MeCN in 20 min. Flow rate was 1 ml/min and temperature 50°C.

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