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Synthesis of new OBAN's and further studies on positioning of the catalytic group †

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Two new zinc ion dependent oligonucleotide based artificial nucleases (OBAN's) have been synthesized. These consist of 2′-*O*-methyl modified RNA oligomers conjugated to 5-amino-2,9-dimethylphenanthroline (neocuproine) *via* a urea linker. OBAN **4** carries the catalytic group on a linker extending from the C-4 of an internal cytosine moiety. OBAN **5** has two neocuproine units attached, each to linkers extending from the C-5 position of uridine moieties, one placed internally and the other at the at the 5′-end of the oligonucleotide. The key step in the synthesis of the OBAN systems is conjugation of the catalytic group to the respective amino linkers of the modified oligonucleotides. This is achieved by first converting the 5-amino-2,9-dimethylphenanthroline to the phenylcarbamate. The reaction of this neocuproine phenylcarbamate with the oligonucleotide carrying one or two primary aliphatic amines in aqueous buffer (at pH 8.5) leads to nearly quantitative formation of the urea-linked conjugates. Both OBAN systems were found to cleave RNA in the bulged out regions formed from the non-complementary part of the target sequences, in the presence of Zn(II) ions. Differences in efficiency between these and previously reported systems are discussed.

Introduction

Oligonucleotide based artificial nucleases (OBAN's) with built in activity to degrade complementary RNA sequences are of current interest in life science and biotechnology.[1] OBAN's may be regarded as a development of traditional antisense methodology where gene expression can be regulated at the level of mRNA by hybridising synthetic oligonucleotides to natural complementary mRNA sequences by means of Watson–Crick base-pairing.[2] Translation may then be inhibited[3,4] and typically catalytic turnover through action of the host RNAse H is beneficial.[4] Chemical modifications of the nucleoside moieties and/or the internucleosidic phosphate linkage are in general necessary to avoid degradation of the antisense oligomer[2] but in most cases these give duplexes where the target RNA is not cleaved by RNase H[2]. The basis for creation of OBAN's, is that the catalytic activity is built into the antisense oligomer by attachment of a catalytic group *via* a linker. Thus, a system with catalytic turnover that is independent on host enzymes can be achieved. Apart from the potential use of OBAN's as mRNA targeting drug substances, these artificial enzymes could also become useful as tools in molecular biology.

In the cleavage of RNA, the nature of the target has been proven to be important. The susceptibility of the RNA to cleavage is sequence dependent.[5] Single stranded RNA and RNA-bulges have proven to be more susceptible to cleavage than duplexes.[6,7] This is suggested to be due to the 2′-hydroxyl in an RNA duplex not being in a suitable orientation for intramolecular transesterification, and a conformational change is energetically unfavourable since the duplex must be partially disrupted.[7] RNA bulges are thus interesting as targets for artificial nucleases and a bulge in the RNA target can be created upon binding of the antisense oligonucleotide by partial non-complementarity of the base sequence.[8,9]

An oligonucleotide based artificial nuclease (OBAN) consists of a recognition element, a modified antisense oligonucleotide, and one or several attached groups that catalyse the hydrolytic cleavage of the RNA target. The catalytic groups are anchored *via* a linker that is intended to place the catalytic group in close proximity to the targeted cleavage site. We have recently developed some OBAN systems where the catalytic group is the Zn(II) complex of 5-amino-2,9-dimethylphenanthroline.[9] Cleavage of simple phosphate esters was first demonstrated by use of a copper complex of the closely related 2,9-dimethylphenanthroline.[10] There have also been a couple of reports that copper and zinc complexes of neocuproine derivatives differently linked to deoxyoligoribonucleotides[11] or PNA[12] (zinc only) can be active in cleavage of RNA, although catalytic turnover was not shown. In our studies, aimed at developing more efficient OBAN systems, we have recently developed 2′-*O*-methyl modified OBAN systems with tethered Zn(II) 5-amino-2,9-dimethylphenanthroline moieties and have shown that these provide turnover of substrate and thus perform real enzyme catalysis in the cleavage of RNA.[9]

For the further development of these artificial enzymes we have begun studies on the influence of linker and linker position as well as of the structure of target RNA on the efficiency of cleavage. We have varied the target so that it forms bulges (or not), of 0–5 nucleotides, when bound to the OBAN. We have also started to evaluate different attachment points for the catalytic group. OBAN **1** from our initial study[9] has the catalytic group linked to C-5 of a deoxyuridine placed centrally in a 2′-*O*-methyl modified RNA sequence (Fig. 1). This OBAN showed the highest activity, especially when the target formed a 3–4-nt bulge upon binding to the OBAN.[9]

In the present study our efficient method for the conjugation of ligand to oligonucleotides in an aqueous environment is described. The method gives consistently nearly quantitative conversions and isolated yields of 80% are generally achieved after purification by RP HPLC. The current study is also a continuation of our efforts to evaluate linkers and synthesis of two new OBAN's are described. These carry 5-amino-2,9-dimethylphenanthroline ligands but linked from other positions and with one new linker arm. This involves synthesis of a novel building block with a linker from the N-4 position of a cytidine moiety, which has a different directionality and length compared to previous linkers. We have also started to evaluate the possibility of concerted action by two neocuproine ligands. The latter system with the ligands extending from the C-5

† Oligonucleotide based artificial nuclease (OBAN) systems. Part. 2. For Part 1 see ref. 9.

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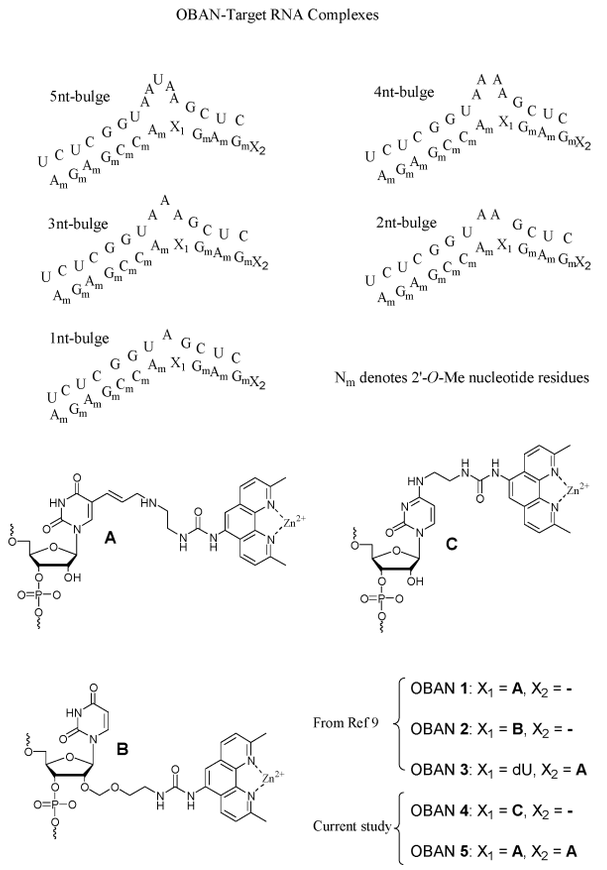


Fig. 1 Illustration of the 1–5-nt bulge systems. Substrate RNA's are written from the 5′-end (from left to right). OBAN **1**,**2** and **4** had either modification **A**, **B** or **C** incorporated at position X_1. OBAN **3** had modification **A** added at X_2. OBAN **5** had two **A** monomers incorporated at positions X_1 and X_2.

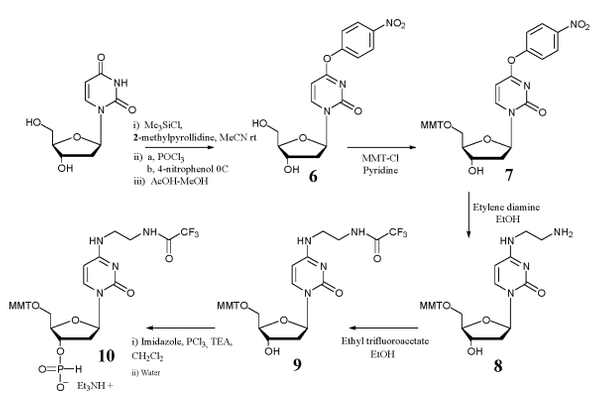
of deoxyuridines, one at the 5′-end and one internally placed (Fig. 1). Results from clevavage of RNA with these systems is compared to the previously developed systems.

Results and discussion

The systems studied here (OBAN **4** and **5**) together with previous constructs (OBAN **1**–**3**) are shown in Fig. 1 Assembly of the modified oligonucleotide leading to OBAN **4** was carried out by standard H-phosphonate methodology on a solid support.[13] A modified deoxycytidine H-phosphonate monomer was used to produce the linker-bearing residue.

Synthesis of the N^4-aminoethyl cytidine building block

This new building block was synthesised from uridine (Scheme 1) *via* the N^4-(4-nitrophenoxy) derivative **6** which was made according to the method developed by Reese and co-workers.[14]

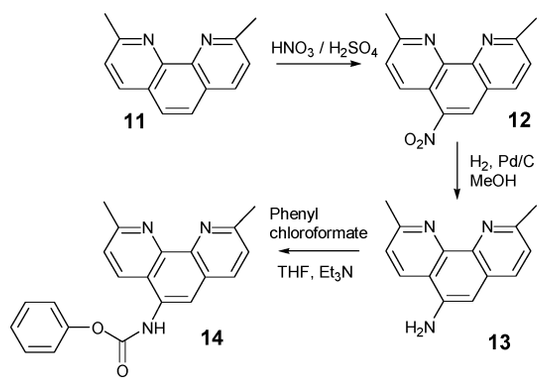


Scheme 1 Synthetic route for the 4-aminoethyl cytidine H-phosphonate building block used for synthesis of OBAN **4**.

The derivative **6** was then treated with 4-monomethoxytrityl chloride in pyridine overnight to produce the 5′-*O*-MMT protected dervative **7**. Subsequent treatment with 8 M ethylenediamine in ethanol afforded the 4-aminoethyl cytidine derivative **8**. We found it more convenient to carry out the synthesis in this order *i.e.* monomethoxytritylation before substitution. When carrying out the synthesis by reacting **6** with ethylenediamine, *i.e.*, without prior tritylation, the conversion to product was satisfactory but due to the high polarity of the compound the purification became somewhat cumbersome. Hence, introduction of the monomethoxytrityl group first gives a more convenient synthesis of **8**. Compound **8** was isolated, after chromatography, in 54% yield over the two steps. The free primary amine of derivative **8** was then protected as the trifluoroacetate (**9**). The protection was performed by reaction of **8** with 1.1 eq. ethyl trifluoroacetate in ethanol at 0 °C, producing compound **9** in 97% isolated yield.[15] Interestingly the NMR spectrum of **9** as recorded from a solution in deuterated chloroform gave doubled signals, presumably from restricted rotation around the amide bond, but change of the solvent to DMSO-d_6 gave only the averaged signals. A plausible explanation could be that the isomerisation rate is lower for the rotamers in chloroform due to a slightly stonger internal hydrogen bond between the 4-NH and the carbonyl oxygen of the trifluoroacetate in the less polar solvent. From **9** the H-phosphonate building block **10** was synthesized by use of the imidazole, PCl_3, triethylamine method.[16] The H-phosphonate **10** was isolated in 67% yield, after chromatography.

Synthesis of the phenylcarbamate reagent of 5-amino-2,9-dimethyl-1,10-phenanthroline

The ligand used, 5-amino-2,9-dimethyl-1,10-phenanthroline was synthesized as described in Scheme 2. 1,10-dimethyl phenanthroline **11** was nitrated to 5-NO_2-2,9-dimethyl-1,10-phenanthroline (**12**) using HNO_3 in oleum at 168 °C.[17] The purification of **12** was complicated due to the multiple by-products that formed during the reaction. Nevertheless, satisfactory amounts of pure **12** could be isolated by silica gel chromatography. **12** was then reduced to 5-amino-2,9-dimethyl 1,10 phenanthroline (**13**) by catalytic hydrogenation;[18] **13** was reacted with phenyl chloroformate at –20 °C overnight whereupon the phenylcarbamate **14** formed as a precipitate.



Scheme 2 Synthetic route for the phenylcarbamate reagent **14**.

A method for conjugation of the neocuproine derivatives to oligonucleotides bearing a free primary amine

Conjugation of the 5-aminoneocuproine ligand to the amino-linker-containing oligonucleotides was carried out on the fully deprotected oligonucleotides in aqueous solution. The conjugation was performed in sodium tetraborate buffer (aq.) at pH 8.5. The reaction of carbamate **14** with the aminolinker-containing oligonucleotides gave virtually quantitative form-

Table 1 Rate constants for cleavage of the RNA substrates in Fig. 1 by OBAN's **1** to **5**

	Previously reported systems[a]			Present systems	
Bulge size	OBAN **1** $k_{obs}/10^6$ s^{-1}	OBAN **2**[9] $k_{obs}/10^6$ s^{-1}	OBAN **3**[9] $k_{obs}/10^6$ s^{-1}	OBAN **4** $k_{obs}/10^6$ s^{-1}	OBAN **5** $k_{obs}/10^6$ s^{-1}
5	9.1 ± 0.2[9] 7.2 ± 0.2[b]	7.5 ± 0.7	5.8 ± 0.1	9.5 ± 0.5	4.4 ± 0.1
4	17.1 ± 0.4[9]	4.8 ± 0.3	5.1 ± 0.1	13.4 ± 0.5	3.3 ± 0.2
3	14.1 ± 0.2[9]	6.2 ± 0.4	4.0 ± 0.1	9.3 ± 0.9	4.6 ± 0.5
2	3.3 ± 0.2[9]	7.5 ± 0.4	2.4 ± 0.1	6.7 ± 0.3	n.d.

Experiments carried out in 10 mM HEPES (pH 7.4), 0.1 M NaCl, substrate RNA's and OBAN's were present at 4 μM, all reactions were incubated at 37 °C. Observed 1st order rate constants were calculated as described in the Experimental section.[a] Data from reference 9, except for the 5A bulge. [b] Result obtained with 5-nt bulge containing 5 adenosine residues (AAAAA) instead of the AAUAA bulge.

ation of the desired conjugates within 2 hours of incubation at ambient temperature (Fig. 2). After purification by RP-HPLC the conjugates were isolated in about 80% yield. Thus, this is a highly efficient method for conjugation to oligonucleotides with aminolinkers in aqueous solution. Post synthetic introduction of the catalytic group gives the advantage that a given oligonucleotide precursor can be reacted with different catalytic groups to produce a plethora of conjugates, and without a need to alter the protocols for oligonucleotide synthesis.

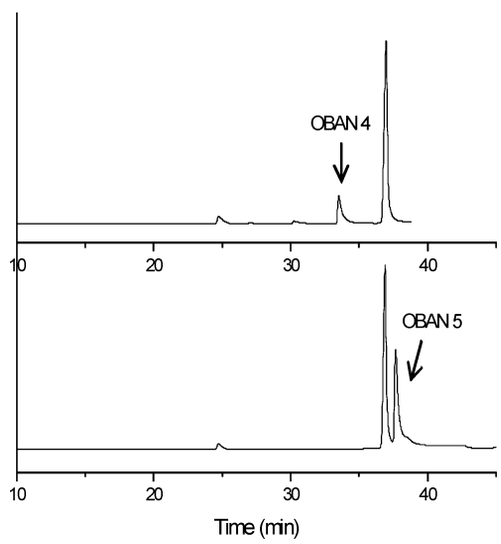


Fig. 2 HPLC chromatograms showing conjugation of the neocuproine unit to the oligonucleotide precursors of OBAN **4** and **5** after 2 h. The reactions were carried out in aqueous sodium tetraborate buffer (pH 8.5). The large residual peak in the chromatograms originates from carbamate **14**. Retention times for non-conjugated OBAN **4** and **5** are 31.13 and 31.23 respectively.

Studies on the cleavage of RNA substrates by OBAN 4 and 5

OBAN **4** in the present study consisted of an 11-mer 2′-*O*-Me modified oligonucleotide carrying a linker at the C-4 position of a central cytosine and OBAN **5** was a 12-mer, conjugated to 2 neocuproines both *via* the C-5 of separate deoxy uridine residues, one additional at the 5′-end and one centrally placed (Fig. 1). Both OBAN's were incubated individually at pH 7.4 with 100 μM Zn^{2+} and 6 different substrate RNA's containing non complementary regions of 0–5 nt to produce 6 different OBAN-RNA complexes for each OBAN. The main goal with the cleavage study was to compare rates of RNA degradation with those obtained for OBAN **1**–**3**. Therefore these conditions were singled out. In our previous study a maximum rate was observed for a 4-nt bulge with OBAN **1**, closely followed by cleavage of the 3-nt bulge. With OBAN **4** we introduce a linker that is one carbon shorter, tethered to the same nucleoside position but with a different directionality. From a 3D-model of the 5-nt bulge it appeared likely that this linker and linker position would be better positioned for cleavage of smaller bulges. As for our previous systems, both the 1-nt bulge and the fully complementary sequence displayed very low rates of cleavage over the time measured and were not further evaluated. For the bulges evaluated, cleavage was exclusively observed in the bulged regions and the bulge size preference for OBAN **4** was indeed shifted towards smaller bulges (Fig. 3, Table 1). The 4-nt bulge is still cleaved with the highest rate but with a lower rate than for OBAN **1**. However, the rate differences between the 2–3-nt bulges and the 4-nt bulge are smaller for OBAN **4** and for the 2-nt bulge the rate is double that for OBAN **1** with the 2-nt bulge. Thus, OBAN **4** is a more efficient cleaver for the 2-nt bulged target and in this respect comparable to OBAN **2**. The linker of the latter does however lead to a higher selectivity for smaller *vs.* larger bulges.

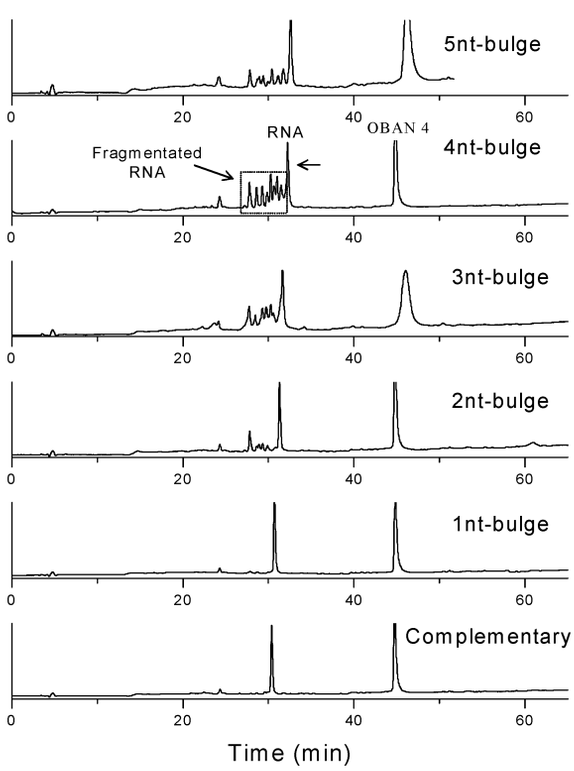


Fig. 3 HPLC chromatograms showing reaction mixtures of OBAN **4** and RNA substrates after 24 h, from the 5-nt bulge forming complex (top) to the complementary duplex (bottom). All reactions were carried out at 37 °C with a 4 μM conc. of both OBAN **4** and substrate RNA, in a 10 mM HEPES buffer (pH 7.4) containing 0.1 M NaCl and 100 μM $Zn(NO_3)_2$. HPLC analysis (see Experimental section) is carried out at pH 6.5.

The sites cleaved by OBAN **4** in the 4-nt bulge system were studied in more detail by LC-MS analysis. As can be seen in Fig. 4, cleavage takes place exclusively in the bulged out region and the extent of cleavage is quite similar between the first 4 phosphates in the bulge (5′ to 3′ direction) while the phosphate between the 4th and the 5th nucleoside is degraded less readily.

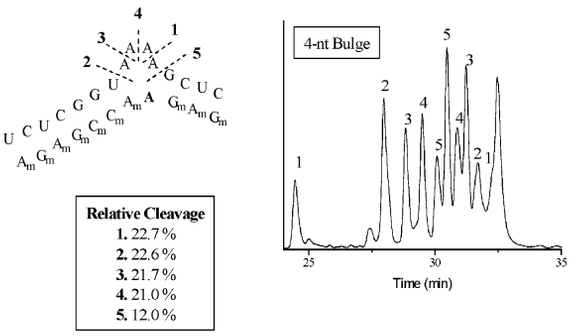


Fig. 4 LC-MS analysis of the fragments formed in cleavage of the 4-nt bulge system using OBAN **4**. The reaction was incubated for 24 h at pH 7.4. Equimolar (4 μM) concentrations of OBAN **4** and substrate RNA were used in a buffer containing 0.1 M NaCl, 10 mM HEPES and 100 μM $Zn(NO_3)_2$. The peak to the far right is intact RNA substrate and the numbered peaks correspond to specific cleavage sites at the bulge. M(−1) for fragments were detected using ESI-TOF MS. **1**: 1530 + 3206, **2**: 2221 + 2516, **3**: 2548 + 2187, **4**: 1859 + 2823, **5**: 1201 + 3536.

Apparently, the linker–catalyst system is equally close in space to first 4 phosphates but still reaches the last phosphate in the bulge. It is notable that there is no selectivity, which is in contrast to the pattern with OBAN **1** that predominantly causes cleavage at positions 3 and 4 (49 and 31% of total cleavage respectively).[9] Fig. 3 indicates that the 5-nt and the 3-nt bulge systems also display cleavage patterns that are essentially non-selective.

It has been shown in studies on dinucleotides that cleavage of ApA in the presence of Cu(II) bi- or terpyridyl chelates can be considerably faster than cleavage of dinucleotides with other base combinations.[19] This apparent base selectivity has been attributed to a π–π interaction between the adenines and the bi or terpyridyl system. If a π–π interation would also be influential in the present case we could obtain a substantial influence on the clevage rate of the 5-nt bulge if the uridine residue in the middle of the bulge is changed to an adenosine. Hence, an oligoribonucleotide forming a 5-nt all adenosine bulge was incubated together with OBAN **1** under indentical conditions to the that for the AAUAA bulge forming substrate. The first order rate constant for the 5-A system was lower and quite similar to that for the AAUAA system. ($7.10 \pm 0.2 \times 10^{-6}$ s^{-1} and $9.1 \pm 0.2 \times 10^{-6}$ s^{-1} respectively). This indicates that there is no strong influence from a π–π interaction between the neocuproine and the adenosine containing bulge. The directionality and length of the linker is clearly more important for positioning of the neocuproine complex.

OBAN **5** carries two catalytic groups in the same oligonucleotide and each respectively linked as in OBAN **1** and **3**. As the catalytic groups of both OBAN **1** and OBAN **3** reach and cleave the bulged regions of the substrate RNA a combination of these could give an additive or even synegistic effect. However, this was not to be. Instead, the RNA cleavage activity of OBAN **5** with dual catalytic groups was lower than for either of the monofunctionalised OBAN's or at best comparable to OBAN **3**. One reason for this could be if binding of the substrate is inhibited, but thermal melting studies confirmed that OBAN **5** also formed stable complexes with the RNA substrates (*e.g.* Tm = 48.2 °C for the 4-nt bulge). A plausible explanation for the low activity with OBAN **5** could be that the two catalytic groups clash sterically, which makes it more difficult for either catalyst to reach the phosphates in a productive orientation. Another possibility is the involvement of an equilibrium where the two ligands also coordinate the same metal ion forming a sandwich complex, which is likely to be inactive. It is however, quite clear that the positions of the catalytic groups are unsuitable and the system perhaps becomes too flexible to obtain a concerted or additive action.

We now have five different oligonucleotides with attached zinc chelates that are cleaving RNA targets. There are marked differences in cleavage rates and patterns. A major factor is likely to be how well the catalytic group is positioned which is also displayed in the differences in substrate (bulge-size) preferences. What is quite clear though is that the difference in absolute numbers is remarkably small. The difference between the highest and lowest rate constants for all systems in cleavage of substrates forming 2–5-nt bulges is just short of an order of magnitude. One plausible explanation for the similarities in rate could be that the bulge region is quite flexible and can be reached quite readily. This flexibility also means that the intramolecularity will be relatively modest. The phosphate–metal interaction is not likely to be strong enough to fix the bulge in a single conformation. This is also supported by the observation of more than a single cleavage site as well as that there is some correlation between site selectivity and overall rate. There are numerous possibilities to vary linkers and linker positions and these may well lead to improvements. However, it does not seem unlikely that these improvements will still be somewhat modest. This reasoning is further justified by comparison with other reported Zn(II) based OBAN's. Komiyama achieved 5% cleavage in 3 h using a binuclear (TPBA) OBAN in 25 eq. excess to substrate RNA and 50 eq. Zn(II).[20] Putnam and Bashkin degraded 25% of a 28-mer and 18% of a 159-mer RNA substrate in 15 and 10 h respectively using a 50 eq. excess of a neocuproine tagged OBAN and the same concentration of Zn(II).[11] Whitney *et al.* conjugated neocuproine to PNA backbones and cleaved 12–30% (internal and external phosphates) of an RNA substrate based on human telomerase in 12–24 h with 50 eq. OBAN and 100 eq. of Zn(II).[12] More recently, Niitymäki *et al.* demonstrated catalytic turnover using an azacrown Zn(II) complex.[21] Substrate was present in 2 fold excess and Zn(II) in 20% excess to OBAN giving a half-life for cleavage of 20 h. With equimolar amounts of our fastest systems (see Table 1), *i.e*, with approximately 80–90% substrate saturation and about 70–80% Zn(II) saturation of the neocuprine complex, we obtain half-lives of 11–14 h. Since the neocuproine complex does not complex strongly to zinc(II) ions the rates at lower zinc concentration are then obviously somewhat lower and the maximum rate at full saturation higher. In any case, the rates for most published systems display a very modest distribution, even though different catalytic groups have been used. A clear possibility to overcome this could be to introduce oligonucleotide modifications that specifically rigidify the bulged out region of the RNA substrate. If this is achieved together with a fairly rigid linker and a productive positioning a much higher intramolecularity is likely to be obtained. This is quite some task to be undertaken, and it will also require good molecular modelling as well as refinement based on structural studies. Our efforts in this direction have only begun, but at least a modest bulge stabilisation of RNA bulges has so far been achieved by introduction of 2′-deoxy-2′-*C*-naphtyl tubercidine in oligonucleotides.[22] We are currently investigating additional bulge stabilising modifications and their use in OBAN systems.

Experimental

Materials

All solvents used were of analytical quality (p.a) and were purchased from Merck (CH_2Cl_2, MeOH, DMSO, THF) and LabScan (pyridine anhydrous) Reagents were purchased from: Merck (Pd/C, oleum), Lancaster (ethylenediamine, imidazole), Aldrich (MMT-Cl) and Acros (phenyl chloroformate).

The ethyl ester of trifluoroacetic acid is synthesised by mixing stoichiometic amounts of ethanol and trifluoroacetic acid. The ester is formed rapidly and can easily be distilled of at 60–62 °C.[15] N^4-(4-Nitrophenoxyl)-2′-deoxy cytosine **6** was synthesized according to a published procedure.[11] The oligonucleotide precursor to OBAN **5** was purchased from TriLink BioTechnologies (San Diego, CA, USA) and purified as

described below. All RNA substrates were purchased from Dharmacon (Boulder, CO, USA). All purchased oligonucleotides were purified by reversed phase HPLC (Hypersil ODS, 250 × 10 mm) using a linear gradient of 0–25% CH_3CN in 50 mM triethylammoniumacetate acetate (pH 6.5). The oligonucleotides were collected, lyophilized, dissolved in water and lyophilized again. Buffers, metal salts and water used for the solutions in the cleavage studies were all of molecular biology grade from Sigma (NaCl, HEPES) or Fluka ($Zn(NO_3)_2$, H_2O).

***N*⁴-(2-aminoethyl)-5′-*O*-(4-monomethoxytrityl)-2′-deoxycytidine 8.** 1.1 g (3.16 mmol) **6** was dissolved in 35 mL dry pyridine and the solvent was evaporated; **6** was then dissolved in another 35 mL portion of dry pyridine and the solution was chilled on an ice-water bath. MMT-Cl (1.07 g, 3.5 mmol) was added under vigorous stirring. The reaction mixture was kept stirring overnight. TLC analysis in CH_2Cl_2 : MeOH 9 : 1 then revealed that all starting material was consumed. 50 μL MeOH was added and the solvent was evaporated under reduced pressure. The crude tritylated derivative was dissolved in 8 M ethylenediamine (in ethanol), 7.9 mL (63.2 mmol). The reaction mixture was stirred for 2 h after which TLC analysis in CH_2Cl_2–MeOH (4 : 1, with 0.1% triethylamine) showed disapperance of the starting material and a product with R_f 0.2. The reaction mixture was, after concentration, partitioned between CH_2Cl_2 and $NaHCO_3$, 100 mL of each. The organic phase was washed two times with $NaHCO_3$ and then with 100 mL water. The organic phase was then dried over Na_2SO_4 and concentrated. The product was purified by chromatography on silica, CH_2Cl_2–MeOH (4 : 1, with 0.1% triethylamine). Fractions containing pure product were pooled, dried over Na_2SO_4 and concentrated. 0.93 g (1.72 mmol) of **8** corresponding to a yield of 54% was obtained. ^{1}H NMR (DMSO-d6): 7.71 (t, 1H, NH C5), 7.58 (d, 1H, C6), 7.24–7.40 (m, 12H, Ar), 6.88 (d, 2H tr), 6.18 (t, 1H, 1′), 5.6 (d, 1H, C5), 5.3 (d, 1H, 3′-OH), 4.25 (m, 1H, 3′), 3.87 (m, 1H, 4′), 3.75 (s, 3H, *O*–Me), 3.22 (m, 4H, 5′ and N4-CH_2), 2.66 (t, 2H, CH_2-N), 2.16 (m, 1H, 2′), 2.04 (m, 1H, 2′). MS (ES/TOF): *m*/*z* = 585.2.

***N*⁴-[2-(2,2,2-trifluoroacetamido)ethyl]-5′-*O*-(4-methoxytrityl)-2′-deoxycytidine 9.** 0.93 g (1.72 mmol) of **8** was dissolved in 40 mL ethanol and the mixture was chilled to 0 °C. 204.6 μL (1.72 mmol) of ethyl trifluoroacetate was added under stirring. After 1 h TLC analysis in CH_2Cl_2 : MeOH 20 : 1 containing 0.05% triethylamine showed completion of reaction and one product with an R_f of 0.3. The reaction mixture was then concentrated, the residue was redissolved in 50 mL CH_2Cl_2, washed with 50 mL $NaHCO_3$, and 50 mL water. The organic phase was then dried over Na_2SO_4 and concentrated. The crude product was purified by chromatography on silica, using CH_2Cl_2–MeOH (20 : 1, with 0.05% triethylamine). Fractions containing pure product were pooled dried over Na_2SO_4 and concentrated. 1.06 g (1.66 mmol) of **9**, corresponding to an isolated yield of 97% was obtained. ^{1}H NMR (DMSO-d6) 9.59 (t, 1H, NH, CF_3CONH), 7.90 (t, 1H, NH, C4), 7.65 (d, 1H, C6), 7.41–7.25 (m, 12H, Ar), 6.91 (d, 2H, tr), 6.22 (t, 1H, 1′), 5.60 (d, 1H, C5), 5.38 (d, 1H, 3′-OH), 4.29 (m, 1H, 3′), 3.91 (m, 1H, 4′), 3.75 (s, 3H, *O*-Me), 3.36–3.44 (m, 4H, CH_2, CH_2), 3.24–3.22 (m, 2H, 5′), 2.21–2.19 (m, 1H, 2′), 2.07–2.03 (m, 1H, 2′). ES/ TOF): *m*/*z* = 639.2

***N*⁴-[2-(2,2,2-trifluoroacetamido)ethyl]-5′-*O*-(4-methoxytrityl)-2′-deoxycytidine 3′-H-phosphonate 10.** Imidazole 0.573 g (8.4 mmol) was dissolved in 30 mL dry CH_2Cl_2 and the mixture was chilled to −10 °C (acetone–dry ice). First PCl_3 (0.239 mL, 2.74 mmol) and then triethylamine (1.22 mL, 8.81 mmol) were added drop-wise under vigorous stirring. The mixture was stirred at −10 °C for 30 min and then cooled to −78 °C. 0.5 g (0.078 mmol) **9** dissolved in 10 mL CH_2Cl_2 was added during a period of 30 min. The reaction mixture was stirred at −78 °C for 1 h and then diluted with 30 mL 2.0 M TEAB aq. pH 7.5. The organic layer was further exracted with 30 mL TEAB buffer. The organic layer was then collected, dried over Na_2SO_4, concentrated and purified on a silica gel column using CH_2Cl_2–MeOH (4 : 1 with 0.1% triethylamine). Fractions containing pure product were pooled and concentrated. After drying under vacuum, 0.58 g (0.072 mmmol) of **10** corresponding to an isolated yield of 92% was obtained. TLC analysis of compound **10** gave: R_f 0.24 (CH_2Cl_2–MeOH, 4 : 1 with 0.1% triethylamine). ^{1}H NMR (DMSO-d6): 7.92 (t, 1H, NH C5), 7.62 (d, 1H, C6), 7.4–7.23 (m, 12.5H, Ar, PH), 6.91 (d, 2H, tr), 6.17 (t, 1H, 1′), 5.85 (s, 0.5H, PH), 5.56 (d, 1H, C5), 4.68 (m, 1H, 3′), 4.05 (m, 1H, 4′), 3.74 (s, 3H, *O*-Me), 3.4 (m, 4H, CH_2, CH_2), 3.28–3.18 (m, 2H, 5′), 2.50 (m, 1H, 2′), 2.14 (m, 1H, 2′). High resolution MS ESI-TOF analysis in negative mode gave *m*/*z* = 701.2012. Calculated *m*/*z* = 701.1988.

5-Nitro-2,9-dimethyl-1,10-phenanthroline[11,23] 12. 2,9-dimethyl-1,10-phenanthroline (**11**), 2g (9.2 mmol), was dissolved in 9.2 ml oleum. 4.8 ml HNO_3 was added and the reaction mixture was heated under stirring to 125 °C (the HNO_3 addition should be carried out at a rate low enough to keep the temperature below 170 °C). After addition was complete the mixture was left stirring for 30 min. Then the mixture was poured onto 130 g of crushed ice. The reaction mixture was treated with 30% NaOH until the solution was neutral. Then dilute nitric acid was added to make the solution slightly acidic, upon which compound **12** precipitated. The crystals were filtered off and washed with water. After drying was the crude product placed in a glass filter funnel and dissolved in chloroform to wash off insoluble byproducts. The filtrate was concentrated and purified on a silica gel column CH_2Cl_2:MeOH (19:1 with 1% triethylamine), R_f 0.33. Fractions containing pure product were pooled, dried over Na_2SO_4 and evaporated. 0.6 g (2.7 mmol) of **12** corresponding to an isolated yield of 29% was obtained. ^{1}H NMR ($CDCl_3$): δ = 8.92 (d, 1H), 8.61 (s, 1H), 8.28 (d, 1H), 7.65 (dd, 2H), 3.01 (s, 1H), 2.99 (s, 1H).

5-Amino-2,9-dimethyl-1,10-phenanthroline[8,23]13. 12 (34.2 mg, 0.14 mmol) was dissolved in 2 ml MeOH and a catalytic amount of Pd/C was added. The reaction mixture was kept under stirring and a H_2 balloon was placed on top of the flask. After 10 min all starting material was consumed and a new product with (R_f 0.33) ($CHCl_3$: MeOH 19 : 1) had been formed. The reaction mixture was filtered and washed with MeOH. The methanol was then evaporated. The crude product was then purified on silica $CHCl_3$: MeOH 4 : 1 containing 0.01% TEA. Fractions containing pure product were pooled and concentrated under reduced pressure. 25.7 mg (0.12 mmol) product corresponding to a yield of 85% was isolated. ^{1}H NMR (CD_3OD): δ = 8.46 (d, 1H), 7.99 (d, 1H), 7.57 (d, 1H), 7.44 (d, 1H), 6.89 (s, 1H), 2.82 (s, 3H), 2.78 (s, 3H).

Phenyl *N*-(2,9-dimethyl[1,10]phenanthrolin-5-yl)carbamate[23]-14. 21.2 mg (0.084 mmol) of **13** was dissolved in 3 ml THF and 17.4 μl triethylamine (0.0921 mmol) was added. The mixture was concentrated to dryness under reduced pressure. 3 mL THF was added together with 17.4 μl triethylamine (0.0921 mmol). Phenyl chloroformate 15.8 μl (0.092 mmol) was then added to the mixture under vigorous stirring. After 30 min TLC (THF) showed disappearence of the starting material and formation of a new product (R_f 0.76). The reaction mixture was then filtered (to remove triethylammonium hydrochloride). The solid on the filter was washed with 1 ml dry THF. The combined filtrates were pooled and placed in a freezer at −20 °C overnight. The precipitated product was washed with cold, dry THF and then dried under reduced pressure. The first crop yielded 10 mg ($2.91 \cdot 10^{-5}$ mol, 35%) of **14**. ^{1}H NMR ($CDCl_3$): δ = 9.01 (d, 1H, H8), 8.85 (s, 1H, NH), 8.18 (d, 1H,

H3), 8.02 (s, 1H, H6), 7.58 (d, 1H, H4), 7.44–7.22 (m, 6H, H7, HPh), 3.12 (s, 3H,CH_3-C2), 2.81 (s, 3H, CH_3-C9) MALDI–TOF MS: m/z = 344.

Oligonucleotide synthesis. The oligonucleotide precursor to OBAN **4** was synthesised with 2′-*O*-Me ribonucleotide H-phosphonate building blocks and the linker containing building block **10** using standard H-phosphonate methodology.[13] Purification by ion exchange HPLC was carried out at 50 °C on a Dionex NucleoPac PA–100 (9 × 250) column, using a linear gradient of 0–90 mM $LiClO_4$ in 20 mM sodium acetate (aq., pH 6.5) containing 30% CH_3CN. The collected fractions were lyophilized and then purified on RP-HPLC as described above for the purchased oligonucleotides. The integrity of the oligonucleotide was confirmed by ESI-TOF MS m/z = 3715.

Conjugation of oligonucleotides to 5-amino 2,9-dimethyl phenanthroline: OBAN 4. 1 mg (2.9 μmol) of carbamate **14** was dissolved in 56 μL dry DMSO. To this solution was added: 28 μL H_2O, 300 μL sodium tetraborate buffer (0.1 M, pH 8.5) and finally a 16 μL (0.1 μmol) water solution of the OBAN **4** precursor. The vial containing the reaction mixture was placed on a shaker, oscillating at low speed; 2 μL aliquots were withdrawn from the reaction mixture, filtrated, diluted with water to 100 μl and analysed by reversed phase HPLC. The reaction was incubated overnight (although reaction appears to be over within 2 h). The reaction mixture was then filtered and OBAN **4** was purified by RP HPLC (as described for all oligonucleotide in the materials section). 0.08 μmol OBAN **4** (as determined by UV) corresponding to a yield of 80% was isolated after two lyophilizations. ES-TOF MS analysis in negative mode gave m/z = 3965.

OBAN 5. 1 mg (2.9 μmol) of carbamate **14** was dissolved in 56 μL dry DMSO. To this solution was added 28 μL H_2O, 300 μL sodium tetraborate buffer (0.1 M, pH 8.5) and finally a 16 μL (0.085 μmol) water solution of the OBAN **5** precursor. The vial containing the reaction mixture was placed on a shaker, oscillating at low speed. 2 μl aliquots were withdrawn from the reaction mixture, filtrated, diluted with water to 100 μl and analysed by reversed phase HPLC. The reaction was incubated overnight. The reaction mixture was then filtered and the product was purified by RP HPLC (see above). 0.068 μmol (as determined by UV) of OBAN **5** corresponding to a yield of 80% was isolated after two lyophilizations. ES-TOF MS analysis in negative mode gave m/z = 4686.

Conditions for RNA cleavage studies. All kinetic runs were performed at t = 37 °C and pH 7.4 (10 mM HEPES buffer containing 0.1 M NaCl and 100 μM $Zn(NO_3)_2$). Reactions were performed with equimolar concentrations of substrate and OBAN, 4 μM respectively, as determind by UV measurements. Concentrations were calculated by the nearest neighbor approach[24] with inclusion of the molar absorptivity constant (ε(260)) for the 5-aminoneocuproine moiety. This was experimentally determined to 16.66 M^{-1} $cm^{-1}\cdot10^{-3}$ by UV measurements of a dilution series H_2O/MeOH (50 : 50) (due to the low solubility of **13** in water). A solution containing 125 μM $Zn(NO_3)_2$, 12.5 μM HEPES buffer and 0.125 M NaCl was filtered through a Millipore Ultrafree-MC 5K centrifugal filter device. 160-μL of this solution was then added to a Wheaton vial. Appropriate amounts of substrate RNA, OBAN were added and then finally water up to a final volume of 0.2 mL. The reaction vial was incubated at 37 °C in a water bath. Immediately after addition of all components as well as at appropriate time intervals 20–30 μL aliquots were withdrawn from the reaction mixture and quenched on a chelating resin (Sigma Chelex 100, iminodiacetic acid). Samples were shaken and left on the resin for 10 min after which they were filtered through a Millipore Millex-GV (4 mm) syringe driven filter unit to remove traces of particles and chelating resin. The aliquots were then diluted to 50 μL with HPLC buffer A (see below) before analysis. The buffers used in the RP HPLC analysis A: 0.1 M triethylammoniumacetate, pH 6.5 and B: 0.1 M triethylammoniumacetate, pH 6.5 containing 50% MeCN. The gradient was used was as follows: 0–5 min: 100% A; 5–40 min 0–30% B; 30–60 min 30–100% B; and the flowrate was 0.2 mL min^{-1}. A Jones Genesis C18 4 μm (250 × 2.1 mm) column was used. LC-MS analysis and calculation of rate constants (first order observed rate constants, *i.e.*, the sum of rate constants for all cleavage sites meaning that the individual constants in, *e.g.*, the system from Fig. 4 for cleavage sites 1–4 is 0.21–0.22·k_{obs}) were carried out as described before.[9]

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