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Directed Crosslinking of RNA by Glutathione-Triggered PNA-Quinone-Methide-Conjugates

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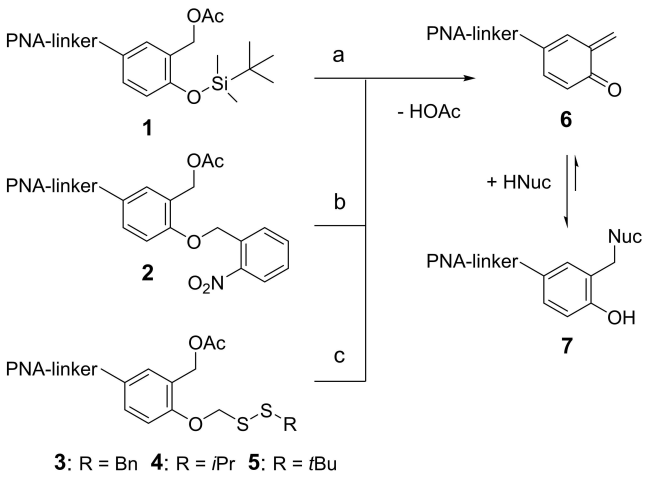
Quinone methide precursors protected with alkyldithiomethyl groups have been synthesized and converted into PNA conjugates. Stable in the absence of reducing agents, the electrophilic quinone methide is released by glutathione in concentrations typical for the cytosol. Self-alkylation then occurs or crosslinking of RNA when hybridized with complementary strands. Fastest reactions are seen for the sterically least hindered compound.

Introduction

Important biochemical techniques are based on crosslinking steps between RNA and bound oligonucleotides or proteins. CLIP (cross-linking immunoprecipitation)[1,2] and HITS-CLIP[3] (or CLIP-seq), for example, are standard methods to localize binding sites of proteins on their target RNA. Notably to map interactions of microRNA-Argonaute complexes with mRNAs, HITS-CLIP is often used.[4,5] Covalent bonds between RNA and the protein part are formed in CLIP by simple UV irradiation. The sensitivity of immunoprecipitation and of high throughput sequencing can compensate the low crosslinking yields in many cases. To improve crosslinking, the PAR-CLIP[6] modification incorporates photoreactive nucleosides such as 4-thiouridine into the mRNA. Alternatively, photochemical [2 + 2] cycloadditions of psoralen-modified miRNAs directly connect them with their mRNA targets (miR-TRAP or mi-CLIP).[7,8] Psoralen-modified anti-miR oligonucleotides showed enhanced inhibitory effects after irradiation.[9] Duplex stabilization of oligonucleotides by covalent crosslinking was also achieved with furanes activated by singlet oxygen,[10,11] light sensitive acetals of unsaturated aldehydes[12] and with 6-vinyl purines.[13,14]

Quinone methides have been intensely studied by Rokita for the directed alkylation of nucleic acids, in particular of DNA.[15–18] Quinone methides are highly reactive electrophiles that cannot be kept in stock. Rokita therefore worked with silyl protected precursors (compound **1** in Scheme 1). Addition of 1 M aqueous fluoride solution liberates the phenolic OH group. The quinone methide **6** is formed by elimination of HOAc which then reacts with nucleophiles leading to **7**. This addition step can be either reversible or permanent, depending on the nature of the nucleophile. Reversible adducts are known for N3 of dC, N1 of dA and N7 of dG whereas NH_2 groups of dA and dG or N1 of dG form stable bonds.[19–23] Because quenching by water is generally slow,[16] intermediate **6** undergoes self-alkylation in the absence of stronger nucleophiles. Such self-adducts are largely reversible over hours and have been used to crosslink complementary oligonucleotides quite effectively.[16] The alkylation of target strands is most efficient, however, with quinone methides released directly before use.



Scheme 1. General structures of quinone methide precursors conjugated with PNA. The silyl protected compound **1** is activated with fluoride[17] (a), compound **2** by irradiation at 365 nm[28] (b), and compounds **3**–**5** by incubation with glutathione (c). Elimination of HOAc generates quinone methide **6** reacting with nucleophiles to form the final product **7**. See Scheme 4 for the PNA sequence present in conjugates **2**–**5**.

An interesting alternative is activation by light.[24–27] In a recent study, we therefore investigated the photolabile precursor **2**.[28] This compound is converted into the quinone methide conjugate **6** by short irradiation at 365 nm. Alkylation of complementary RNA strands then occurs in yields up to 70%. Although the photochemical release of electrophile **6** has the advantage of spatiotemporal control, it is less appropriate for systemic use. Drug-like applications would prefer a stable quinone methide precursor which upon cell entry is converted into **6** by the reducing environment of the cytosol. The redox potential of cells is mainly defined by the large excess of free

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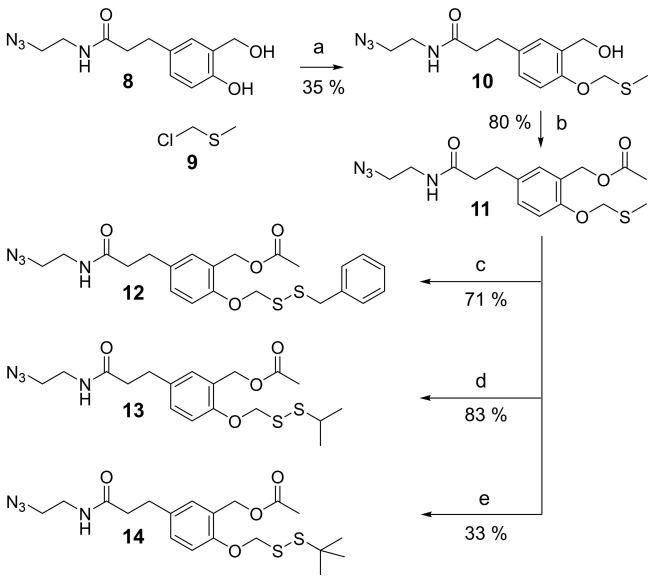
glutathione (GSH) over its disulfide form (GSSG). Whereas extracellular concentrations of GSH are in the low micromolar range, 0.5–10 mM are present in the cytosol.[29] GSH is often used for the intracellular cleavage of disulfide linkers or disulfide based protecting groups.[30–35] A first DNA-quinone methide conjugate activated by reduction was already described in 1991.[36] However, $NaBH_4$ or NADH in combination with cytochrome c reductase were required for reduction. GSH was found to be insufficient. More recently, the formation of *p*-thioquinone methides from appropriate sulfonated precursors could be triggered by GSH.[37] On the other hand, nucleophilic addition of thiols might also quench the quinone methide and prevent the desired crosslinking of RNA.[24–26,38–40] To find a proper balance between GSH induced activation and quenching rates, we synthesized the precursor compounds **12**–**14** and conjugates **3**–**5** characterized by disulfide protecting groups of increasing steric demand. Thus, S–S cleavage by nucleophilic attack should be fastest in disulfide **3** and slowest in compound **5**. Subsequent elimination of thioformaldehyde and of HOAc was expected in all cases to end up in the same quinone methide structure **6**. An azido linker was chosen for conjugation with alkyne modified peptide nucleic acids[41] (PNA) as published previously.[28]

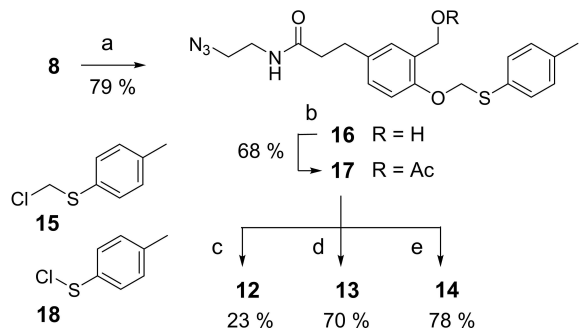
Results and Discussion

The *tert*-butyldithiomethyl group (DTM) present in compound **5** was first described as a 2′-OH protection in the chemical synthesis of RNA.[42] A similar group was also used by Ono.[32] The synthesis of compounds **12**–**14** (Scheme 2), starting from the known building block **8**,[28] is mainly based on this methodology. Selective alkylation of the phenolic OH group with **9**[43] (**10**, 35%) and acetylation afforded the common precursor **11** (80%). To convert **11** into product **12**, reaction with SO_2Cl_2 first replaced the $-SCH_3$ group by chlorine. Cl was substituted next by thiotosylate and in the final step benzyl mercaptan formed the desired S–S bond with tosylsulfinate serving as a leaving group. All three reactions were conducted in a single step and yielded 71% of compound **12**. Compounds **13** (83%) and **14** (33%) were prepared by analogous procedures. The latter reaction has not been optimized and the observed yield is probably below the limit. However, we could not improve the poor and variable yield for the alkylation of **8** with **9**, although good to excellent results have been reported for similar cases.[42,44–45] The reaction of **8** with chloromethylarylsulfide **15**,[46,47] in contrast, reliably led to compound **16** (79%) that could be transformed into quinone methide precursors **12**–**14** as before (Scheme 3). We found beneficial, however, to add cyclohexene as a scavenger of sulfenyl chloride **18** which is formed in the chlorination step with SO_2Cl_2.[42] Reduction of azides by thiols, although possible in general, did not affect the synthesis of **12**–**14**.

With quinone methide precursors **12**–**14** in hands, the 1,3-dipolar cycloaddition with an alkyne modified PNA strand[28] was accomplished in the presence of Cu^{2+}-TBTA and ascorbic acid (Scheme 4). Although this step seemed critical, cleavage of disulfide bonds was not observed. After successful conjugation, the products were purified by HPLC. When incubated at pH 7 and 37 °C for 72 h in the absence of thiols, none of the three conjugates showed signs of decomposition (Figure S4). We studied next their behavior in the presence of low concentrations of GSH (0.5 mM) but without RNA (Figure 1). With compound **3**, the most reactive conjugate, a faster running peak could be observed already after 15 min. Such peaks have been assigned previously to products of self-alkylation.[28] The HPLC pattern changed with time because metastable intermediates are slowly converted into the final alkylation products.[28] The decay of **3** as a function of time matched first order kinetics with $k = 3.2 \pm 0.6\ h^{-1}$. Conjugates **4** and **5** behaved in the same way qualitatively but reacted slower with increasing steric demand of the disulfide substituent (Table 1



Scheme 2. Synthesis of the redox-labile quinone methide precursors **12**–**14**. a) KO*t*Bu, NaI, DMF/THF, then **9**, 35%; b) Ac_2O, pyridine, 80%; c) Et_3N, SO_2Cl_2, CH_2Cl_2, then potassium thiotosylate, then benzyl mercaptan, 71%; d) as before but with isopropyl mercaptan, 83%; e) as before but with *tert*-butyl mercaptan, 33%.



Scheme 3. Alternative synthesis of compounds **12**–**14**. a) KO*t*Bu, CH_3CN/THF, then **15**, 79%; b) Ac_2O, pyridine, 68%; c) Et_3N, SO_2Cl_2, CH_2Cl_2, then cyclohexene, then potassium thiotosylate, then benzyl mercaptan, 23%; d) as before but with isopropyl mercaptan, 70%; e) as before but with *tert*-butyl mercaptan, 78%.

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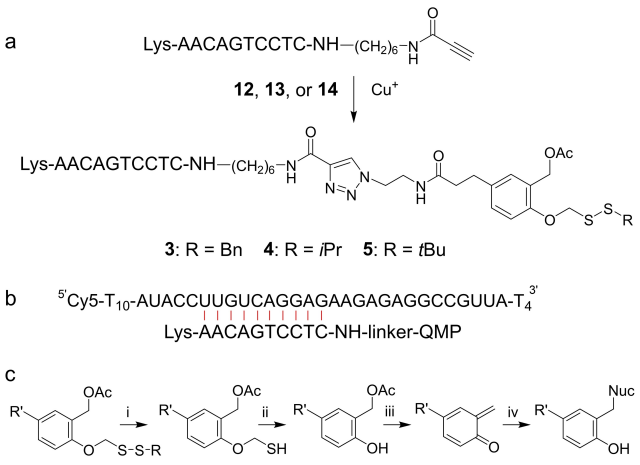

Scheme 4. a) Conjugation of the alkyne modified PNA strand and quinone methide precursors (QMP) **12**, **13**, or **14**. b) Hybrid of PNA conjugates bound to their dye-labeled RNA substrate. c) Formation and trapping of quinone methides. i: Reductive cleavage of the disulfide. ii: Elimination of H_2CS. iii: Elimination of HOAc. iv: Addition of NucH.

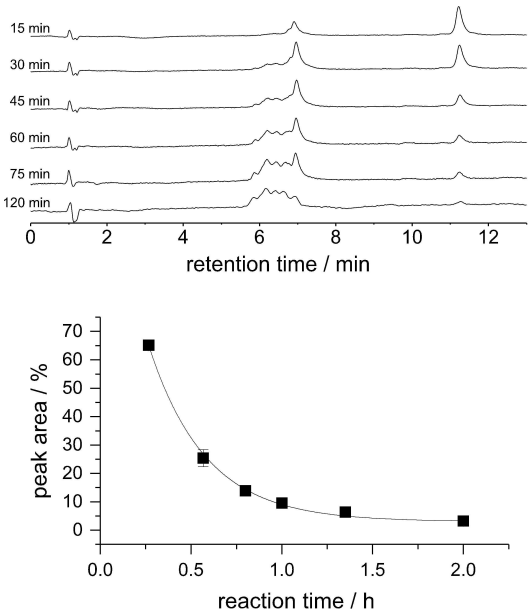

Figure 1. Top: HPLC analysis of reductive activation of conjugate **3** (20 μM) in the presence of 0.5 mM GSH (37 °C, 130 mM MES buffer pH 7.0, 130 mM NaCl, see also Figure S4). Starting material appears at 11.3 min, the complex mixture of self-alkylation products around 6 min. Bottom: Decay of [**3**] as a function of time and best fit to a first order rate model.

Table 1. Decay of PNA conjugates at low and at high GSH concentrations, determined by HPLC as shown in Figure 1.

[GSH]	**3**	**4**	**5**
0.5 mM	$3.2 \pm 0.6\ h^{-1}$	$0.23 \pm 0.05\ h^{-1}$	$0.025 \pm 0.005\ h^{-1}$
10 mM	$> 10\ h^{-1}$	$2.0 \pm 0.4\ h^{-1}$	$0.33 \pm 0.06\ h^{-1}$

and Figures S5–S6). Thus, replacing the benzyl group in compound **3** by *tert*-butyl lowered the rate of reductive activation by roughly two orders of magnitude. At higher GSH concentrations of 10 mM, the decay of **3** became too fast to be recorded in our assay. For **4** and **5** rate constants of 2.0 h^{-1} and 0.33 h^{-1} were observed (Table 1). In contrast to the complex pattern of self-alkylation products visible in Figure 1, a dominating peak with a retention time around 5.9 min appeared in all reactions with high GSH concentrations (Figures S7–S8). This peak also became visible after extended incubation of conjugate **4** (Figure S5) with 0.5 mM GSH (see also Figure S6). Isolation by HPLC and MALDI mass spectra identified it as the covalent GSH adduct of the quinone methide (Figures S9–S10). This observation shows that GSH can directly quench the quinone methide but may also transform parts of the metastable self-adducts.

To study crosslinking, the PNA conjugates **2**–**5** were hybridized with a complementary dye-labeled RNA strand (Scheme 4b) and then activated with light[28] or incubated with GSH at concentrations of 0.5 or 10 mM. Identical oligonucleotide sequences and linker structures were used in all experiments. We then separated the RNA substrate and the crosslinked products by gel electrophoresis in an ALFexpress DNA sequencer.[28] All dye-labeled components induced fluorescent signals in proportion to their relative concentrations (Figure 2) allowing us to determine alkylation rates and yields. The peak of unmodified RNA appeared at 116 min. Crosslinking with PNA increased the mass but not the charge of the RNA strand and thus shifted the retention time to 135–175 min. In absence of GSH, however, crosslinking was not observed even with the most reactive conjugate **3** (Figure 2a). A low gel temperature of 25 °C was used in all electrophoretic runs to record stable and metastable bonds simultaneously.[28] The time course of crosslinking is shown in Figure 3.

When conjugate **2**[28] was irradiated for 2 min at 365 nm in the presence of complementary RNA (Scheme 4b), fast alkylation and a final crosslinking yield of 70 % were observed (black squares in Figure 3). Red dots show the analogous process

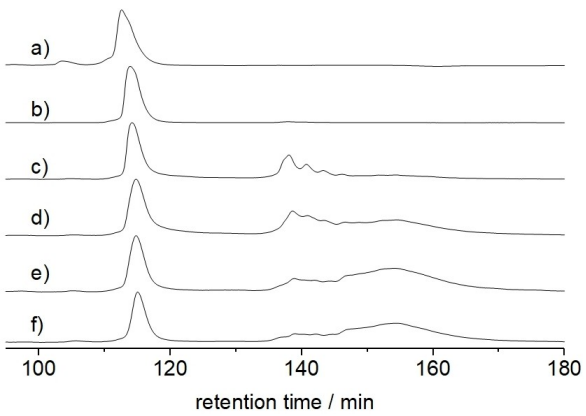

Figure 2. Alkylation of the dye-labeled RNA substrate (3 μM) by conjugate **3** (6 μM) in the presence of 0.5 mM GSH (37 °C, 130 mM MES buffer pH 7.0, 130 mM NaCl). Analysis by gel electrophoresis using the ALFexpress DNA sequencer. The RNA substrate appears at 116 min, the crosslinking products in the region from 135 to 175 min. Peak areas are proportional to the concentration of substances. a) Incubation of RNA with conjugate **3** for 48 h in absence of GSH. b) RNA before incubation. c) Incubation of RNA with conjugate **3** and 0.5 mM GSH after 4 h, 8 h (d), 24 h (e), and 48 h (f).

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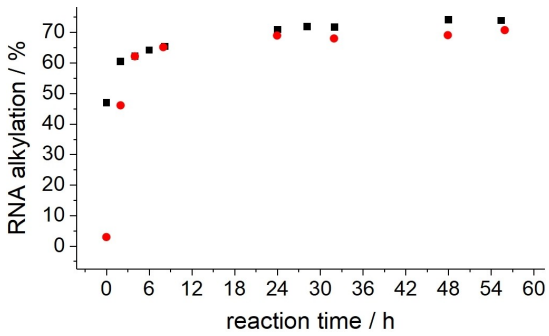

Figure 3. Comparison of RNA alkylation (3 μM) by freshly irradiated PNA conjugate **2** (6 μM, black squares)[28] and by conjugate **3** (6 μM, red dots) after activation with 0.5 mM GSH (37 °C, 130 mM MES buffer pH 7.0, 130 mM NaCl). The time course of both reactions and the final crosslinking yields are very similar.

when conjugate **3** reacted with RNA in the presence of 0.5 mM GSH. Within the first 3 h crosslinking was slightly retarded because redox activation of **3** is slower than light induced deprotection of **2**. The final yield of alkylation products, however, is very similar in both reactions. Thus, 0.5 mM of GSH is sufficient to activate conjugate **3** but does not significantly quench the quinone methide intermediate (for redox-activation with *N*-acetyl cysteine see Figure S12). Figure 4 compares RNA alkylation by conjugates **3**, **4**, and **5**. As expected, increasing steric demand of the protective group slowed down alkylation but also reduced the total crosslinking yield, hardly exceeding 20% in the case of **5** even after 72 h. The results are summarized in Table 2.

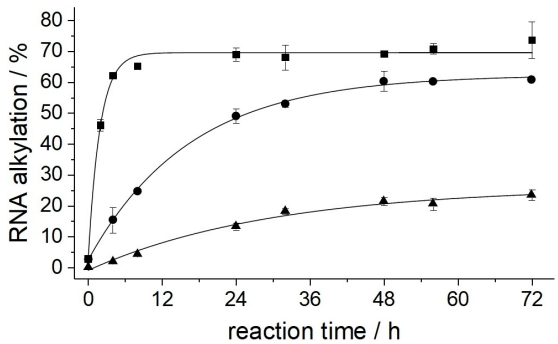

Figure 4. Comparison of RNA alkylation by conjugate **3** (squares), by conjugate **4** (dots), and by conjugate **5** (triangles), each after activation with 0.5 mM GSH (3 μM RNA, 6 μM conjugate, 37 °C, 130 mM MES buffer pH 7.0, 130 mM NaCl). Data points are fitted against a single exponential function.

Table 2. Crosslinking rates and yields at low and at high GSH concentrations, determined by gel electrophoresis as shown in Figure 2. Yields determined after 72 h (0.5 mM GSH) and 48 h (10 mM GSH) at 37 °C.

[GSH]	3	4	5
0.5 mM	$0.53 \pm 0.1\ h^{-1}$	$0.058 \pm 0.012\ h^{-1}$	$0.033 \pm 0.006\ h^{-1}$
yield (72 h)	71%	60%	23%
10 mM	$> 10\ h^{-1}$	$0.43 \pm 0.08\ h^{-1}$	$0.23 \pm 0.05\ h^{-1}$
yield (48 h)	43%	37%	23%

Incubation with 10 mM of GSH, representing the upper limit in cells, accelerated the crosslinking of RNA (Figure S11). With conjugate **3**, the reaction became too fast to determine the rate constant. The maximum of yield was already observed after few minutes but it dropped from 71 to 43%. Even the slowest reaction with conjugate **5** was largely completed after 9 h. The yield, however, did not exceed 23%. The lower crosslinking yields at high GSH concentrations presumably result from partial quenching of the quinone methide intermediate.

Conclusion

Our previous studies with compound **2** and related PNA conjugates of light sensitive quinone methide precursors have shown that crosslinking is specific for complementary RNA strands (for additional experiments see Figure S14). In accordance with Rokita's observations, alkylation occurs preferentially at sites rich in purines, in particular in adenosine. The RNA sequence used in this study is a favorable substrate leading to high crosslinking yields. In absence of GSH, all conjugates **3**–**5** are stable and even the most reactive compound **3** does not spontaneously alkylate the bound RNA strand. Upon addition of 0.5 mM GSH, however, crosslinking by conjugate **3** is as effective as alkylation by the photolabile analog **2**. Partial quenching of quinone methides by GSH becomes relevant only at glutathione concentrations of 10 mM, representing the extreme upper limit in cells. Reductive activation of quinone methide precursors thus should be useful to trigger the crosslinking of nucleic acids at cellular GSH concentrations. Possible applications may comprise the trapping of miRNA-Argonaute complexes and their redirection to novel target sites in non-cognate mRNAs.[48]

Experimental Section

HPLC analysis of the reductive activation of conjugates 3–5 with glutathione (Figure 1). A 20 μM solution of conjugate **3**, **4**, or **5** (130 mM MES buffer pH 7.0, 130 mM NaCl) was incubated with 0.5 mM or 10 mM GSH in a polyethylene vial (total volume 140 μL) and kept at 37 °C. Samples of 20 μL were taken at the time given in Figure 1 and analyzed by HPLC. To determine the rate of reductive activation, the peak area of the unchanged conjugate was divided by the sum of all peaks visible between 5 and 12 min. The peaks between 5 and 8 min represent the self-adducts but also the O,S-acetal, the free phenol, and the GSH-adduct. HPLC conditions used for Figure 1, Figures S4–5, S7–9, and S13: Phenomenex Gemini C18, 150×4.6, 5 μm, linear gradient from 7–40% MeCN in 0.1 M TEAA buffer (pH 7.0) for 10 min, 2 mL/min, 50 °C, 260 nm. HPLC conditions used for Figure S6: Phenomenex Gemini C18, 150×4.6, 5 μm, 2 min hold at 4% MeCN, linear gradient of 4–35% MeCN in 0.1% TFA for 25 min, 1 mL/min, 50 °C, 260 nm.

RNA crosslinking by conjugates 3–5 after reductive activation with glutathione (Figures 2–4). A solution of conjugates **3**, **4**, or **5** (6 μM) and Cy5 labeled RNA (3 μM; see Scheme 4b) in MES buffer (pH 7.0, 130 mM NaCl) was incubated with 0.5 mM or 10 mM GSH in a polyethylene vial (LoBind; total volume 20 μL) and kept at 37 °C. Samples of 1 μL were taken, diluted with sterile DEPC treated water to a final volume of 10 μL and kept at −20 °C. Analysis of the

samples by denaturing PAGE (16% acryl amide, 7 M urea; 150× 310 mm or 270×310 mm plates) was performed on a DNA sequencer (ALFexpress™, GE Healthcare/Amersham Bioscience). Prior to analysis, all samples were diluted with loading buffer (8 M urea, 20 mM EDTA and 0.2% crocein orange in DEPC treated water) to a final RNA concentration of 150 nM. The gel cassette of the sequencer was filled with 1×TBE buffer and 5 μL of each sample was loaded onto the gel. Conditions of electrophoresis: 1500 V (maximum), 60 mA (maximum), 25 W (constant), 25 °C, 2 s sampling interval. The electropherogram was then analyzed with the AlleleLinks 1.01 software package (Amersham Biosciences, Uppsala, Sweden).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Antisense agents · Alkylation of RNA · Disulfide bond · Glutathione · Peptide nucleic acids

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