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Site specific labelling of sugar residues in oligoribonucleotides: reactions of aliphatic isocyanates with 2′ amino groups

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ABSTRACT

Considerable effort has been directed towards studying the structure and function of nucleic acids and several approaches rely on the attachment of reporter groups or reactive functional groups to nucleic acids. We report here the selective modification of 2′-amino groups in oligoribonucleotides, through their reaction with aliphatic isocyanates, to give the corresponding 2′-urea derivatives in >95% yield. Furthermore, such modification with (2-isocyanato)ethyl 2-pyridyl disulfide enables subsequent coupling to other thiols (such as those contained in peptides and proteins) or to thiol-reactive electrophiles. A modified decamer was not significantly destabilized by the 2′-urea group, compared with a 2′-amino group, as demonstrated by a mere 1.3°C drop in the melting temperature of the duplex.

INTRODUCTION

The conjugation of reporter groups and reactive groups to oligonucleotides represents a useful approach for studying the structure and function of nucleic acids (for recent reviews see 1–3). The attachment of such groups onto oligonucleotides can be achieved either by their incorporation during chemical synthesis or by post-synthetic modification. In the latter case it is often desirable to have an oligonucleotide with a reactive functional group, such as an amine or a thiol, which must be introduced during oligonucleotide synthesis. One of the advantages of post-synthetic labelling is that there is a wide choice of compounds that can be attached to the oligonucleotides and the choice of compound is not restricted by the availability of phosphoramidites. Furthermore, the functional groups to be incorporated might be incompatible with the conditions used in oligonucleotide synthesis.

Although there are numerous examples of post-synthetic labelling at the ends of oligonucleotides, relatively few examples have been reported for modification at internal positions (4–10). One of these approaches utilizes the incorporation of 2′-amino groups into oligonucleotides (11,12), which can be selectively reacted with aromatic isothiocyanates to form 2′-thiourea-modified oligomers (6,7). This method was recently utilized for the site-specific introduction of two thiol groups into the hammerhead ribozyme to probe its tertiary structure by disulphide-mediated cross-linking (13,14). The drawback of this method is that an aromatic thiourea substituent could cause structural perturbations in a complex three-dimensional RNA structure due to both its size and inflexibility. To avoid these potential complications in our continued efforts towards establishing tertiary interactions within ribozymes, we were interested in incorporating smaller and more flexible aliphatic groups at internal 2′ positions of such catalytic oligoribonucleic acids. We report here that aliphatic isocyanates, such as methylbenzylisocyanate and (2-isocyanato)ethyl 2-pyridyl disulfide (isocyanate **1**), react selectively and in high yield with 2′-amino groups in oligoribonucleotides and describe the preparation and characterization of such a modified oligoribonucleotide.

RESULTS

The aliphatic isocyanate **1** (Fig. 1) was synthesized using a slightly modified procedure from our previously published method for the efficient synthesis of isocyanates from aliphatic amines (15). When the 2′-amino-containing oligoribonucleotide **I** was reacted with isocyanate **1** (Figure. 2), under the same conditions as previously utilized for modification with aromatic isothiocyanates (13), complete conversion to oligomer **II** was obtained. However, a control oligomer lacking a 2′-amino group (GCCGACCGACAUU) also yielded minor products, indicating non-specific side reactions of the isocyanate with the oligoribonucleotide. The reaction conditions (pH, temperature and concentration of isocyanate) were therefore optimized such that the 2′-amino group was selectively modified without concurrent side reactions. Interestingly, the yield of the 2′-modified oligomer **II** increased upon lowering the temperature, presumably due to repression of competing isocyanate hydrolysis. Thus, incubation of oligomer **I** (1 mM) with isocyanate **1** (15 mM) at pH 8.6 and 0°C gave 96% conversion to oligomer **II**, whereas an oligomer lacking the 2′-amino group was unchanged, as monitored by HPLC (Fig. 3). Selective modification of oligomer **I** with the secondary isocyanate *R*-(+)-α-methylbenzylisocyanate was achieved in >95% yield (data not shown). For further characterization of the 2′-urea-derivatized oligomer **II**, its preparation was scaled up, followed by HPLC purification. Scaling up the reaction required

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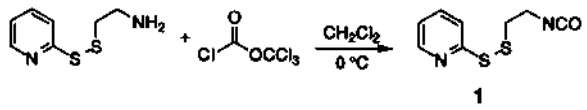

Figure 1. Synthesis of isocyanate **1**.

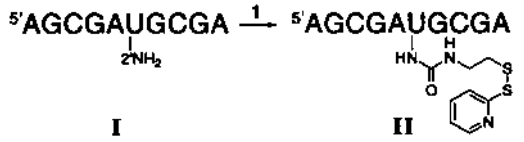

Figure 2. Reaction of oligomer **I** with isocyanate **1** to yield the 2′-urea-modified oligomer **II**.

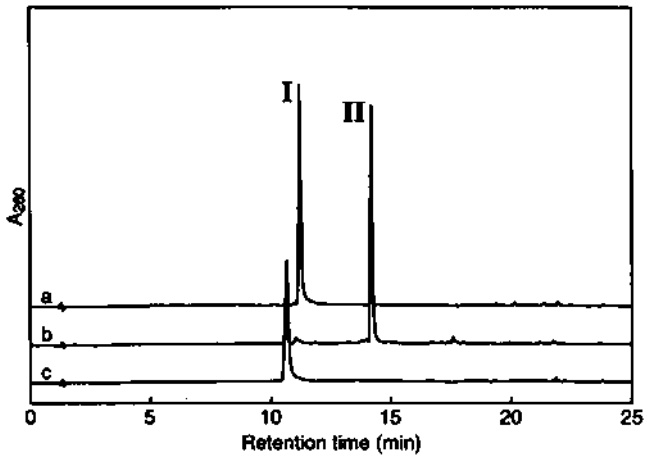

Figure 3. Monitoring the reaction of isocyanate **1** with oligomers by HPLC. Trace a, oligomer **I** [5′-AGCGA(2′NH2U)GCGA]; trace b, after reaction of oligomer **I** with isocyanate **1** to yield oligomer **II**; trace c, after incubation of the oligomer 5′-GCCGACCGACAUU with isocyanate **1**, which does not yield detectable products.

either a second addition of isocyanate **1** or the reaction to be performed at lower temperatures (–8°C) to give comparable yields to that of the analytical reactions.

For analysis, oligomers **I** and **II** were digested with snake venom phosphodiesterase and calf spleen alkaline phosphatase (Fig. 4). The fact that 2′-aminouridine was no longer present in the digest of oligomer **II**, in conjunction with the appearance of the strongly retained substance **2** (HPLC analysis), is consistent with the isocyanate reacting with the 2′-amino group in oligomer **I**. To verify the proposed structure of uridine derivative **2**, its synthesis was undertaken by reaction of isocyanate **1** with 2′-aminouridine (Fig. 5). The presence of the 2′-urea functionality was demonstrated by a 2D-COSY NMR experiment, which showed that one of the urea protons was coupled to the 2′-proton of the sugar residue and the other was coupled to one of the disulphide methylenes. HPLC analysis of an admixed sample of the oligomer **II** digest and synthetic **2** confirmed the identity of the compounds (data not shown).

To investigate the possible effects of the newly introduced 2′-urea functionality on duplex stability, oligomers **I** and **II** were

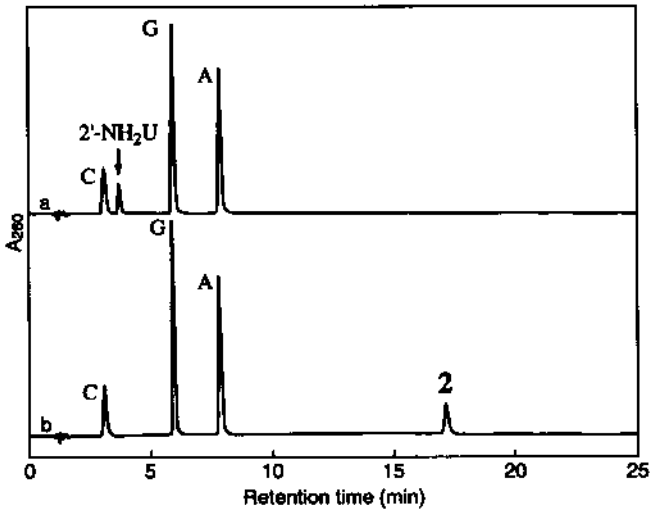

Figure 4. HPLC profiles of oligomers **I** and **II** (traces a and b respectively) after digestion with snake venom phosphodiesterase and alkaline phosphatase.

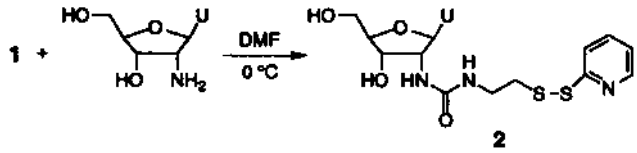

Figure 5. Synthesis of substance **2**.

annealed to their complementary sequence (5′-UCGCAUCGCU) and the absorbance at 260 nm recorded as a function of temperature. The melting temperature of the duplex containing oligomer **II** was determined to be 62.9°C, 1.3°C lower than the corresponding duplex of oligomer **I**, indicating only minor destabilization of the duplex structure by introduction of the 2′-urea group.

Oligomer **II** was reacted further for additional verification of the presence of the disulphide functionality and to establish its reactivity (Fig. 6) and the reactions monitored by HPLC (Fig. 7). Oligomer **II** (trace a) was first incubated with DTT to give the thiol-containing oligomer **III** (trace b). Subsequent incubation of this crude reaction mixture with an excess of bromobimane yielded oligomer **IV** (trace c). For conjugation of thiols to oligoribonucleotides, oligomer **II** (trace a) was incubated with ~1 equivalent of glutathione and resulted in a complete conversion to oligomer **V**, with the concomitant release of 2-mercaptopyridine (trace d).

DISCUSSION

The simplest experimental approach for derivatizing oligonucleotides is to attach the reporter or reactive functional groups at either the 3′- or 5′-terminus, for which a variety of methods are available (for reviews see 1–3). However, it is often desirable to introduce probes at internal positions in nucleic acids to investigate their interactions with other macromolecules. The probes can be linked either to the nucleotide bases or the sugar–phosphate backbone, which minimizes disruption of base

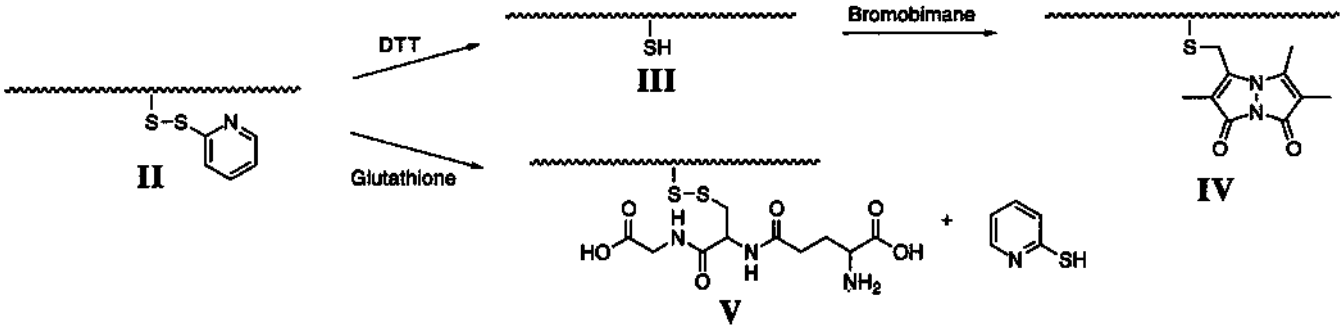

Figure 6. Schematic representation of modifications of oligomer **II** by either a reduction of the disulphide bond (oligomer **III**) followed by reaction with an electrophile (oligomer **IV**) or by reaction with a thiol to form a mixed disulphide (oligomer **V**).

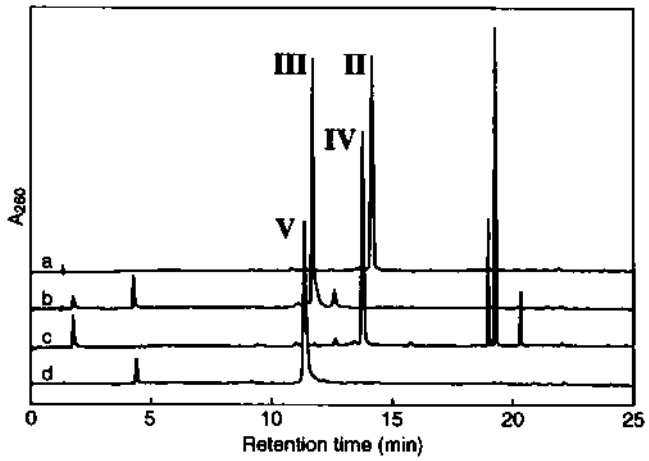

Figure 7. HPLC analyses of the reactions of oligomer **II**. Trace a, oligomer **II**; trace b, after treatment of oligomer **II** with DTT to form oligomer **III**; trace c, after reaction of oligomer **III** with bromobimane to give oligomer **IV**; trace d, after incubation of oligomer **II** with glutathione for formation of oligomer **V**. The peak at ~4.2 min in traces b and d represents 2-mercaptopyridine. The peaks appearing between 18 and 21 min in trace c are excess bromobimane and presumably its adducts with 2-mercaptopyridine and DTT.

pairing interactions. One method has been to label 2′-hydroxyl groups by the use of modified phosphoramidites during oligomer synthesis. Sproat and co-workers have stabilized RNA molecules against nuclease degradation by alkylation of 2′-hydroxyl groups (16,17), as well as linking an ethylene group containing a terminal thiol for post-synthetic labelling (18). The 2′-hydroxyl group has also been utilized for tethering anthracene at internal positions in oligomers (19) and for installing an amino group, through a five carbon atom tether, for post-synthetic labelling (20). Modified phosphoramidites have also been used for the incorporation of amides (21,22) at the 2′-position of oligoribonucleotides.

Alternatively, oligomers containing 2′-amino groups (11,12) can be post-synthetically modified by reaction with aromatic isothiocyanates, to yield the corresponding 2′-thiourea derivatives (6,7,13). Attempts to extend this approach to aliphatic groups by the reaction of 2′-amino-containing oligomers with succinimidyl esters (7) or aliphatic isothiocyanates (data not shown) have met with limited success. We have shown here that the 2′-amino group in oligoribonucleotides reacts readily with aliphatic isocyanates to yield a 2′-urea derivative. Characterization of oligomer **II** by enzymatic digestion and comparison of the modified nucleotide with an authentic sample is fully consistent with the structure depicted in Figure 2. Thus, post-synthetic labelling of the sugar–phosphate backbone in oligoribonucleic acids can be accomplished by reaction of 2′-amino groups with either aromatic isothiocyanates or aliphatic isocyanates and complements the labelling of nucleotide bases by the convertible nucleoside approach of Verdine and co-workers (8,10).

An important criterion for practical oligomer labelling is the effect of the newly introduced functionality on duplex stability. It has been determined that the introduction of 2′-amino groups into RNA oligomers results in a moderate decrease in duplex stability; the melting temperature of an RNA duplex was 4.3°C lower after introduction of the modification, corresponding to the energy of a hydrogen bond (7). We have shown here that subsequent modification of the 2′-amino group with isocyanate **1** results in a further decrease of only 1.3°C. These destabilizing effects are of similar magnitude to those reported for other internal labels at either the sugar or base moieties of oligonucleotides (4,7,21), with the exception of intercalating agents, which can stabilize duplex structure (19).

To further the scope of this labelling strategy to molecules containing functional groups other than aliphatic isocyanates we have used isocyanate **1**, which serves as a molecular adapter for introducing an activated (protected) thiol. Reduction of the disulphide in oligomer **II** yielded the free thiol **III**, which can participate in nucleophilic substitution and addition reactions with a variety of functional groups (23), as illustrated here by reaction with bromobimane (24) (Figs 6 and 7). Thiols can also be used to form disulphides, which is useful for thiol-mediated cross-linking (13,25,26) and for the reversible incorporation of labels into oligonucleotides. The thiol-protecting group in isocyanate **1** serves to activate the thiol for formation of mixed disulphides after its incorporation into oligoribonucleotides (27). This approach has found use in the formation of oligonucleotide conjugates with proteins (28) and peptides (29,30) and is demonstrated here by the efficient reaction of glutathione with oligomer **II** to form oligomer conjugate **V** (Figs 6 and 7).

CONCLUSIONS

We have shown that aliphatic isocyanates react selectively with 2′-amino groups in oligoribonucleotides to form 2′-urea derivatives in yields >95%. The introduction of this modification

causes only a minor destabilization of the duplex structure. This strategy, coupled with our recent method for the efficient preparation of aliphatic isocyanates containing sensitive functionalities (15), provides a general approach for the site-specific labelling of RNA using compounds that contain aliphatic amines. In addition, the use of isocyanate **1** expands the range of functionalities for incorporating labels into oligomers to thiols and thiol-reactive electrophiles.

MATERIALS AND METHODS

General

Syntheses were carried out under a positive pressure of argon. 2′-Aminouridine was purchased from Amersham International, trichloromethyl chloroformate and *R*-(+)-α-methylbenzylisocyanate from Aldrich Chemical Co., bromobimane from Sigma and glutathione (reduced form) from Merck. Snake venom phosphodiesterase (*Crotalus durissus*) and calf spleen alkaline phosphatase were purchased from Boeringer Mannheim. Flash column chromatography was performed on silica gel 60 (Merck) with a particle size of 0.04–0.063 mm. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker AM 360L instrument at 360.13 and 90.55 MHz respectively. Chemical shifts are reported in p.p.m. and coupling constants (J) in Hz. High resolution, accurate mass spectra (HRMS) were recorded on a on VG Analytical Autospec-T tandem mass spectrometer using electron impact ionization.

Oligoribonucleotides were prepared by automated chemical synthesis using phosphoramidites from MilliGen/Biosearch, except for the incorporation of trifluoroacetyl-protected 2′-amino-modified nucleotides (11). Deprotection and purification of oligoribonucleotides was performed as previously described (31). Concentrations of oligomers were calculated using a molar extinction coefficient of 6600 $M^{-1}cm^{-1}$/nucleotide, except for preparations of solutions for melting experiments (see below).

HPLC analyses were carried out on a Waters Associates System with Model 6000A pumps, a Model 680 Automated Gradient Controller, a Model 730 Data Module and a Model 481 LC Spectrophotometer. Separations by HPLC were performed using reverse phase ODS Hypersil (5 µm; Shandon). Solvent gradients for analytical HPLC were run at 2 ml/min. Elution was performed with a linear gradient of 100 mM triethylammonium acetate, pH 7.0, containing from 0 to 16% CH_3CN over 15 min, followed by an increase to 70% CH_3CN over 5 min, which was then maintained for 10 min, with a subsequent return to the original conditions (0% CH_3CN) over 3 min. For preparative HPLC purification of modified oligomers, the following modifications to the analytical conditions were made: flow-rate was 4 ml/min, 100 mM triethylammonium bicarbonate, pH 7.0, was used instead of 100 mM triethylammonium acetate, pH 7.0, and during the elution the conditions were left at 16% CH_3CN for 5 min.

Syntheses

*(2-Isocyanato)ethyl 2-pyridyl disulphide (**1**).* (2-Isocyanato)ethyl 2-pyridyl disulphide was synthesized using a slightly modified procedure from that previously reported (15). *S*-(2-Pyridyldithio)cysteamine hydrochloride 3 (0.100 g, 0.449 mmol) was partitioned between CH_2Cl_2 (1.5 ml) and 1 M NaOH (1 ml). The organic phase was separated and dried (Na_2SO_4) (note that this material decomposes upon concentration of the solution) and added drop-wise to a stirred solution of trichloromethyl chloroformate (0.022 g, 0.11 mmol) in CH_2Cl_2 (1 ml) at 0°C over a period of 1 min. After stirring for 2 min at 0°C, the suspension was partitioned between 1 M HCl (5 ml) and CH_2Cl_2 (10 ml), after which the organic phase was separated and washed successively with 1 M HCl (1 ml) and 1 M NaOH (1 ml). After drying the organic phase (Na_2SO_4), the solvent was removed *in vacuo* to yield **1** as a pale yellow oil (0.024 g, 25% based on the starting amine), which was ~98% pure as determined by ^{1}H NMR analysis.

We observed that **1** slowly hydrolyses (~30% after 4 weeks) when stored concentrated at –20°C. However, when it was stored desiccated at –20°C, as 1 mg aliquots in 100 µl CH_2Cl_2, the isocyanate was still intact after 3 months. The solvent was removed *in vacuo* prior to reaction with the oligomers.

*Substance **2**.* A suspension of 2′-aminouridine (0.100 g, 0.411 mmol) in DMF (0.5 ml) was treated with a solution of **1** in DMF (0.5 ml) and stirred at 25°C for 30 min. The solvent was removed *in vacuo* and the product was purified by flash column chromatography (5% MeOH/CH_2Cl_2) to yield **2** as a colourless oil (0.177 g, 94%). ^{1}H NMR (DMSO-d_6; DMSO-d_5 as an internal standard at δ 2.50 p.p.m.): δ 2.81 (2H, t, J=6.6, SCH_2), 3.21 (2H, m, NHC**H**$_2$), 3.56 (2H, m, H5′), 3.90 (1H, m, H4′), 4.02 (1H, m, H3′), 4.29 (1H, m, H2′), 5.16 (1H, t, J=5.0, 5′-OH), 5.65 (1H, d, J=8.1, H5), 5.81 (1H, d, J=14.6, H1′), 5.82 (1H, s, 3′-OH), 6.13 (1H, d, J=8.7, 2′-NH), 6.58 (1H, t, J=5.7, CH_2N**H**), 7.24 (1H, m, ArH), 7.79 (2H, m, ArH), 7.86 (1H, d, J=8.1, H6), 8.45 (1H, m, ArH), 11.25 (1H, s, H3). ^{13}C NMR (DMSO-d_6; DMSO-d_5 as an internal standard at δ 39.5 p.p.m.): δ 38.4, 38.7, 55.3, 61.9, 70.9, 86.4, 86.8, 102.1, 119.3, 121.2, 137.8, 141.0, 149.6, 151.1, 157.5, 159.3, 163.3. HRMS: 456.1006 (calculated 456.1011 for $C_{17}H_{21}N_5O_6S_2$).

Reactions of oligoribonucleotides with isocyanates

Reactions of isocyanates with oligoribonucleotides on an analytical scale were carried out in an ice bath in a cold room (6°C). A solution of oligomer **I** (2 mM in 2.5 µl 70 mM borate buffer, pH 8.6) was treated sequentially with DMF (2.0 µl) and **1** in DMF (150 mM, 0.5 µl) or *R*-(+)-α-methylbenzylisocyanate in DMF (100 mM, 0.5 µl) and incubated for 3.5 h. For HPLC analyses, 1.5 µl aliquots of reaction mixture were diluted into 20 µl triethylammonium acetate buffer (100 mM, pH 7.0) and extracted with CH_2Cl_2 (2 × 150 µl) to remove the organic material.

Large scale preparation of oligomer **II** was carried out in an ice bath. To a solution of oligomer **I** [5′-AGCGA(2′-NH_2U)GCGA] (2 mM in 233 µl 70 mM borate buffer, pH 8.6) was added **1** in DMF (30 mM, 233 µl), followed by purification of the modified oligomer by HPLC. This reaction resulted in 78% conversion to product, but a second addition of the isocyanate or carrying out the reactions at –8°C resulted in comparable yields to those obtained in the analytical reactions.

Enzymatic digestion of oligomers

Enzymatic digestion of the oligoribonucleotides was essentially performed as described by Connolly (33). A solution of the oligoribonucleotides (0.2 mM) in Tris buffer (56 µl, 50 mM, pH 8.0) containing $MgCl_2$ (10 mM) at 37°C was treated sequentially with snake venom phosphodiesterase (6 µl, 0.003 U/µl) (incubation for

5 h) and alkaline phosphatase (6 µl, 1 U/µl) (further incubation at 37°C for 0.5 h) followed by HPLC analysis of the digest.

Melting curves

Absorption measurements (260 nm) for generating melting curves were recorded with a Uvicon 820 spectrophotometer (Kontron, Zürich, Switzerland) at 1°C intervals. The molar extinction coefficients were calculated for oligomer **I** [5′-AGCGA(2′-NH_2U)GCGA; 107×10^3 $M^{-1}cm^{-1}$) and the complementary sequence (5′-UCGCAUCGCU; 92×10^3 $M^{-1}cm^{-1}$) using extinction coefficients of nucleotides and dinucleotides (34). The additional contribution of the thiopyridyl moiety to the extinction coefficient of oligomer **II** was estimated to be 3000 $M^{-1}cm^{-1}$ by comparing the integrated areas under the peaks of 2′-NH_2U and **2** in the HPLC traces of the enzymatic digests of oligomers **I** and **II** respectively. The oligomers (2 µM) in sodium cacodylate buffer (10 mM, pH 7.0) containing EDTA (1 mM) and NaCl (1 M) were incubated for 3 min at 56°C and cooled slowly to 25°C in order to ensure complete hybridization of the two strands before recording the absorbance versus temperature.

Reactions of disulphide-containing oligomers

Reactions with bromobimane. To a solution of oligomer **II** (2 mM in 4 µl 50 mM HEPES buffer, pH 8.0, 5 mM EDTA) was added DTT (10 mM, 3 µl), followed by incubation under argon at 37°C for 1.5 h, after which HPLC analysis indicated that reduction was complete. An aliquot (2 µl) of this solution was added to a solution of bromobimane (20 mM in 2 µl 50% CH_3CN–25 mM HEPES, pH 8.0, 2.5 mM EDTA) and incubated in the dark at 25°C for 1 h, followed by HPLC analysis.

Reactions with glutathione. The disulphide-containing oligomer **II** (0.9 mM) was incubated with the reduced form of glutathione (1.8 mM) in borate buffer (5 µl, 35 mM, pH 8.6) under argon at 25°C for 2.5 h, followed by HPLC analysis.

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