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A versatile post-synthetic method on a solid support for the synthesis of RNA containing reduction-responsive modifications†

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An original post-synthetic method on a solid support was developed to introduce various disulfide bond containing groups at the 2′-OH of oligoribonucleotides (RNAs). It is based on a thiol disulfide exchange reaction between several readily accessible alkyldisulfanyl-pyridine derivatives and 2′-*O*-acetylthiomethyl RNA in the presence of butylamine. By this strategy, diverse 2′-*O*-alkyldithiomethyl RNAs were obtained. These modifications provided high nuclease resistance to RNA and were easily removed with glutathione treatment, thus featuring a potential use for siRNA prodrugs.

Introduction

Small interfering RNAs (siRNAs) have attracted considerable attention because they play an important role in gene silencing strategies.[1,2] However, their poor cell permeation and their low resistance to nucleases remain the major hurdles to their development as therapeutics. To circumvent these problems, chemists introduced several permanent chemical modifications into siRNAs.[3,4] Despite an increase of the enzymatic stability of siRNA, it is difficult to predict the influence of the permanent modifications on siRNA activity which can significantly reduce the gene knockdown efficiency. Another way to overcome this drawback is to use temporary biolabile modifications which are designed to be removed inside cells to liberate the active siRNA. Over the last few years, our research has focused on a prodrug-based approach using 2′-*O*-acetalester groups at 2′-OH of RNAs, which should be removed by carboxyesterases. These transient modifications increased the siRNA stability towards enzymes, enhanced their cellular uptake and promoted gene silencing after spontaneous naked delivery.[5–8] In the same way, recently Dowdy and colleagues evaluated short interfering ribonucleic neutrals (siRNNs) whose internucleoside backbones containing S-acetyl-2-thioethyl (SATE) phosphotriester groups[9] are unmasked inside the cells by thioesterases.[10] The results obtained with the above described strategies are very encouraging and show that prodrug approaches are promising for the development of siRNA therapeutics.[11] Among other possible transient modifications, biolabile groups containing a disulfide link are likewise attractive in prodrug strategies.[12–15] Indeed, disulfide bonds are reduced to thiol groups under reducing conditions such as in the presence of glutathione whose intracellular concentration ranges from 1 mM to 10 mM whereas the extracellular concentration is low.[16,17] Such biolabile modifications might also confer nuclease resistance to siRNA and enhance their cellular uptake. Thus, we developed a facile chemical synthesis to introduce various alkyldithiomethyl (RSSM) groups containing either lipophilic moieties or polar groups at 2′-OH of RNA (Fig. 1). These modifications were designed to be cleaved in a two-step process: (1) the reduction of the disulfide bond leading to an unstable thiohemiformacetal, (2) the release of a

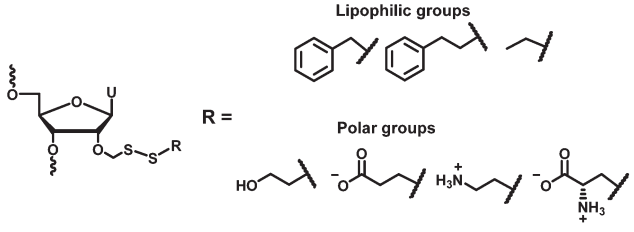

Fig. 1 Alkyldithiomethyl (RSSM) modifications at 2′-OH of RNA.

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†Electronic supplementary information (ESI) available: NMR spectra of uridine phosphoramidite **1** and alkyldisulfanyl pyridine reagents, analytical data of RNAs, T_m data and CD spectra of RNA duplexes, MALDI-TOF MS spectra with SVPDE, and IEX-HPLC chromatograms with glutathione. See DOI: 10.1039/c6ob01272h

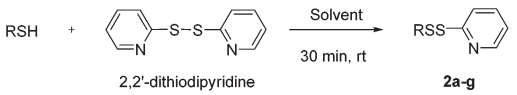
thioformaldehyde generating the native RNA. As far as we know, only a few examples of 2′-*O*-RSSM RNAs have been reported. First, the *tert*-butyldithiomethyl (DTM) group was described as a 2′-*O*-protecting group for RNA synthesis.[18,19] However, due to the instability of the disulfide bond in a 2′-*O*-DTM phosphoramidite derivative, because the phosphite triester moiety can attack the disulfide bond intramolecularly, the use of this protection was rather limited.[20] To avoid this unwanted reaction an elegant post-synthetic approach was proposed for the synthesis of 2′-*O*-methyldithiomethyl (2′-*O*-MeSSM) oligonucleotides which were obtained by the conversion in a solution of appropriate 2′-*O*-(2,4,6-trimethoxybenzylthiomethyl) oligonucleotides upon treatment with dimethyl-(methylthio)-sulfonium tetrafluoroborate.[21] The weakness of this post-synthetic approach is that exclusively the 2′-*O*-MeSSM modification can be incorporated into RNA. We now report a robust versatile post-synthetic method on a solid support leading readily to diverse modified 2′-*O*-alkyldithiomethyl (2′-*O*-RSSM)/2′-OH RNAs. Different RSSM modifications were shown to increase RNA resistance to degradation by a 3′-exonuclease and to be efficiently converted into 2′-OH under reducing conditions.

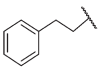
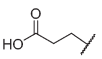
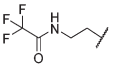
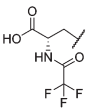
Results and discussion

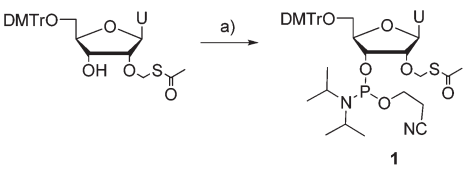
Synthesis of 2′-*O*-RSSM oligonucleotides

To form the disulfide bond, our method consists of a thiol–disulfide exchange between a thiolate anion and an alkyldisulfanyl-pyridine (RSSPy) derivative. This thiol–disulfide exchange is rapid and irreversible because the pyridinesulfenyl group is a good leaving group.[22,23] The thiolate anion was generated under basic conditions from the deprotection of a 2′-*O*-acetylthiomethyl (2′-*O*-AcSM) derivative previously studied in our lab (Scheme 1).[8] In the partially modified 2′-*O*-RSSM/2′-OH RNAs, the 2′-OH resulted from the removal of 2′-*O*-pivaloyloxymethyl (2′-*O*-PivOM) groups under the standard ammonia treatment.[24] Therefore, the synthesis of 2′-*O*-RSSM/2′-OH modified RNAs required the use of commercially available 2′-*O*-PivOM phosphoramidites, 2′-*O*-AcSM uridine phosphoramidite **1** and RSSPy derivatives (Table 1). The 2′-*O*-AcSM protected amidite uridine **1** was isolated with high purity in very good yield (90%) after the 3′-phosphitylation of 2′-*O*-acetylthiomethyl-5′-*O*-(4,4′-dimethoxytrityl)-uridine (Scheme 2).[8]

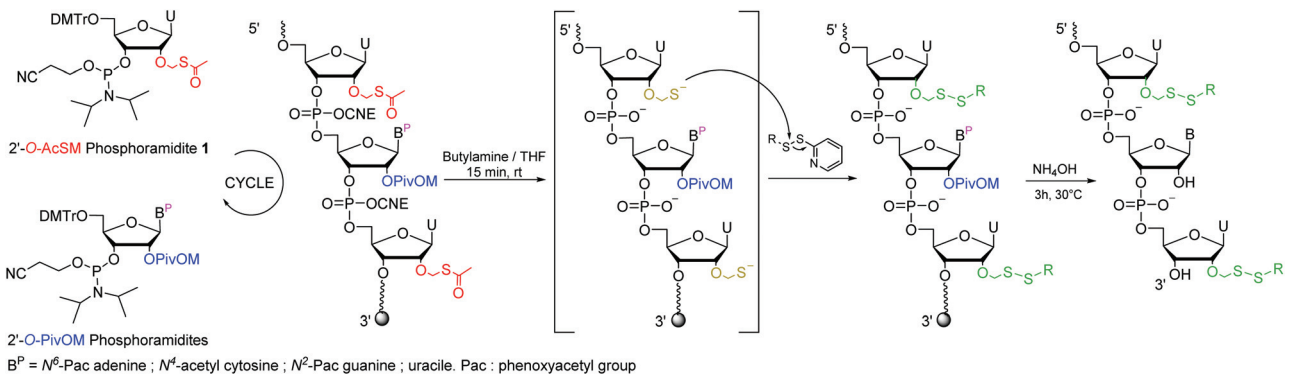
Table 1 Synthesis of RSSPy derivatives **2a–g**



No.	R group	Solvent	Yield (%)
2a		MeOH/DMF 1/1	81
2b		MeOH/DMF 1/1	68
2c		MeOH	77
2d		MeOH	61
2e		MeOH	56
2f		MeOH/DMF 1/1	77
2g		MeOH	43



Scheme 2 Synthesis of 2′-*O*-AcSM uridine phosphoramidite unit **1**. Reagents and conditions: (a) $(iPr)_2NP(Cl)OCNE$, DIEA, DCM, rt, 2.5 h, 90%.



Scheme 1 Synthetic strategy for 2′-*O*-RSSM/2′OH RNAs.

The RSSPy derivatives were prepared by the reaction of diverse commercially available thiols (RSH) with 2,2′-dithiodipyridine in methanol or a mixture of MeOH/DMF according to a described procedure (Table 1).[25,26]

Thus, some RSSPy reagents bearing different chemical functionalities (aromatic (**2a**, **2b**), aliphatic (**2d**), alcohol (**2c**), carboxylic acid (**2e**), amine (**2f**) and amino acid (**2g**)) were obtained with satisfactory yields (from 43 to 81%).

The trifluoroacetyl group was used to protect the amino function of RSSPy derivatives **2f** and **2g** to avoid side reactions during the synthesis of 2′-*O*-RSSM/2′-OH RNAs. This protecting group remained stable during the formation of the disulfide link and was removed under standard ammonia deprotection. No protection was needed for alcohol (**2c**) and carboxylic acid (**2e**, **2g**) functions.

Several 21-mer RNAs **4a–g** (Table 2) corresponding to the sense strand of a siRNA targeting *ICAM-1* mRNA with four RSSM groups spread out over the sequence were synthesized on an automated DNA synthesizer with uridine amidite **1** and 2′-*O*-PivOM phosphoramidites by using commercially available controlled-pore glass (LCAA-CPG) linked to 5′-*O*-DMTr-dT through a 3′-*O*-succinyl linker. The elongation of the RNAs was carried out on a 1 μmol scale, following a published automated RNA synthetic procedure with a 180 s coupling step and 5-benzylmercaptotetrazole (BMT) as the activator.[24] At the end of the elongation, thiol–disulfide exchange was performed by a treatment of 2′-*O*-AcSM/2′-*O*-PivOM RNAs still "on support" with RSSPy derivatives **2a–g** (100 equivalents per modification) and an anhydrous solution of butylamine in THF for 15 min at room temperature. During this reaction, the cyanoethyl groups protecting the phosphates were also removed. Then, in a second step, an aqueous concentrated ammonia treatment was applied at 30 °C for 3 h to remove nucleobase protection and 2′-*O*-PivOM groups, and release 2′-*O*-RSSM/2′-OH RNAs **4a–g** from the solid support. Crude RNAs **4a–g** were analyzed and purified by IEX-HPLC and characterized by MALDI-TOF mass spectrometry (Table 2 and ESI, Fig. S9–S15†). They were efficiently obtained with satisfactory yields (45–65%) and high purities (92–98%) (Fig. 2 and Table 2).

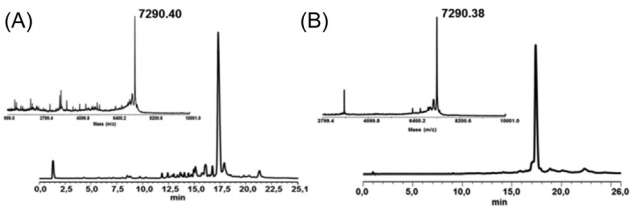


Fig. 2 IEX-HPLC and MALDI-TOF spectra of (A) crude and (B) purified RNA **4b**.

Table 2 Data of 2′-*O*-RSSM/2′-OH RNAs

Sequence 5′ → 3′ [a] $GCCU_{RSSM}CAGCACGU_{RSSM}ACCU_{RSSM}CU_{RSSM}AdTdT$ [b]

RNA	R group	RNA of interest [c] (%)	MALDI-TOF MS [d] Calcd	MALDI-TOF MS [d] Found	Purity of RNA [e] (%)	T_m [f] (°C)	ΔT_m [g] (°C)	Time of RNA degradation [i] (min)
3 [h]	None	71	6564.73	6563.16	100	81.7	—	7
4a		65	7236.58	7235.76	92	79.7	−2.0	>20
4b		62	7292.02	7290.38	92	79.7	−2.0	>20
4c		61	7044.15	7044.07	95	80.7	−1.0	15
4d		59	6988.22	6987.87	95	79.7	−2.0	20
4e		64	7164.59	7163.64	97	79.7	−2.0	15
4f		45	7048.52	7048.36	98	80.7	−1.0	>20
4g		58	7224.41	7224.00	98	80.7	−1.0	>20

[a] U_{RSSM} = 2′-*O*-RSSM uridine; G, C, A, U = 2′-OH nucleosides. [b] Sense strand of siRNA targeting *ICAM-1* mRNA. [c] Percentages of RNA of interest present in the crude samples after RNA deprotection determined by IEX-HPLC. [d] Negative mode. [e] Purity of oligonucleotides after purification by IEX-HPLC. [f] T_m values obtained from UV melting curves at 260 nm with 3 μM strand concentration in 10 mM sodium cacodylate, 100 mM NaCl, pH 7.0. Data are the average of three hybridization-melting cycles. Estimated error in T_m = ±0.5 °C. Complementary RNA strand: *UAGAGGUAC-GUGCUGAGGCdTdT*. [g] ΔT_m is the difference in T_m relative to the 2′-OH unmodified duplex. [h] Unmodified sense strand of siRNA targeting *ICAM-1* mRNA. [i] Nuclease stability of RNAs towards SVPDE determined by MALDI-TOF Mass Spectrometry. Time was determined when the main mass peak corresponded to a 4-mer.

Thermal stabilities

The influence of the different RSSM modifications on the thermal stability of duplexes formed between RNAs **4a–g** and their complementary unmodified strand was evaluated by UV-melting experiments at 260 nm (Table 2 and ESI, Fig. S16†). Compared to the unmodified duplex with RNA **3** (T_m = 81.7 °C), all duplexes containing 2′-*O*-RSSM groups were slightly destabilized (ΔT_m = −1.0 °C to −2.0 °C). Nevertheless, these destabilizations ($\Delta T_m \leq -0.5$ °C per modification) were not detrimental to the formation of stable duplexes under physiological conditions. In addition, circular dichroism (CD) spectra of all modified duplexes indicated the typical curve of an A-form helix geometry with a positive band near 264 nm and a strong negative band at 210 nm (ESI, Fig. S17†). The shapes of the CD spectra were similar to that of the unmodified duplex. Therefore, whatever the RSSM modification the original A-form conformation of the RNA duplex was maintained.

Nuclease resistance

The stability of 2′-*O*-RSSM/2′-OH RNAs **4a–g** towards a 3′-exonuclease (snake venom phosphodiesterase (SVPDE)) was evaluated by MALDI-TOF mass spectroscopy (Table 2 and ESI, Fig. S18†).[27] All the 2′-*O*-RSSM/2′-OH RNAs **4a–g** exhibited resistance to SVPDE compared to unmodified RNA **3** which was degraded within 7 min. It is noteworthy that a correlation between the RNA degradation in the presence of SVPDE and the nature of the RSSM group can be established. Indeed, the more lipophilic the RSSM group, the more stable the RNA towards SVPDE.[28] At 20 min, RNAs bearing alcohol (**4c**), carboxylic acid (**4e**) and aliphatic moieties (**4d**) were nearly totally degraded whereas for RNAs with aromatic groups (**4a** and **4b**) a peak corresponding to a 10-mer was still noticeable in the mass spectrum (ESI, Fig. S18†). Moreover, RNAs with RSSM containing amine groups (**4f** and **4g**), protonated at physiological pH, had a similar stability to RNAs bearing aromatic groups (**4a** and **4b**) (Table 2). This relative stability could be due to the presence of positive charges, which induce unfavorable interactions with the SVPDE metallic ion essential for the enzyme activity.[29,30]

Glutathione treatment

To use 2′-*O*-RSSM/2′-OH modified siRNAs in a prodrug approach, the disulfide bond of 2′-*O*-RSSM groups should be converted into 2′-OH in an intracellular reductive environment. First, the reductive conversion of 2′-*O*-RSSM RNA **4a** into the corresponding 2′-OH RNA **3** was evaluated in 5.6 mM glutathione in 7.5 mM HEPES buffer (pH 8) and monitored by IEX-HPLC. After 1 h incubation, in the HPLC chromatogram, the new major peak was assigned to the 2′-OH RNA **3** but a shoulder peak was also noticeable. The MALDI-TOF mass analysis showed that this peak corresponds to the hemithioacetal species resulting from the disulfide bond cleavage and the subsequent incomplete degradation of the unstable intermediate within 1 h of incubation (ESI, Fig. S19†). The same experiment was conducted with 2′-*O*-RSSM duplexes with RNAs **4a–g**.

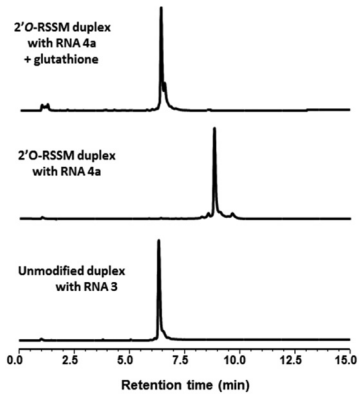


Fig. 3 IEX-HPLC analysis of the reductive conversion of the duplex with RNA **4a** into the unmodified duplex with RNA **3** by treatment with 5.6 mM glutathione in 7.5 mM HEPES buffer (pH 8) for 1 h.

After 1 h incubation, as shown in Fig. 3 for the duplex with RNA **4a**, all the peaks corresponding to the disulfide-containing duplexes have disappeared and the native 2′-OH duplex was mainly generated (with 70 to 95% purity). A shoulder peak corresponding to the thiohemiformacetal intermediate was also noticeable in the chromatograms for all RSSM groups (ESI, Fig. S20 and Table S1†). The disulfide bond reduction has proceeded rapidly and cleanly whereas the complete conversion into 2′-OH RNA has required a longer time under these experimental conditions.

This preliminary study performed under physiological conditions anticipates that all 2′-*O*-RSSM groups should be efficiently reduced within the cells releasing the unmodified 2′-OH duplex.

Conclusions

We developed an original and straightforward post-synthetic method on a solid support, to introduce efficiently a large variety of alkyldithiomethyl groups at the 2′-OH of RNA. This method is versatile since from one 2′-*O*-AcSM-containing RNA, we can obtain numerous 2′-*O*-RSSM-modified RNAs bearing lipophilic or polar groups depending on the alkyldisulfanylpyridine derivative used for the disulfide–thiol exchange. The results demonstrated that RSSM modifications did not disrupt the duplex stability dramatically while maintaining an A-form conformation and they provided RNA resistance against 3′-exonuclease. These features are promising for designing effective siRNA constructs. In addition, an efficient and rapid conversion of 2′-*O*-RSSM groups into 2′-OH under reducing conditions similar to those found in the intracellular medium shows the great potential of 2′-*O*-RSSM/2′-OH RNAs for their use as prodrugs of siRNA. To extend the scope of applications of 2′-*O*-RSSM RNAs and for the design of potent siRNAs, the synthesis of other 2′-*O*-AcSM phosphoramidite ribonucleotides is in progress. We anticipate that this post-synthetic method would be also attractive in the context of RNA cross-linking with other biomolecules (nucleic acids, peptides, proteins, sugars).

Experimental section

General

CH_3CN, MeOH, pyridine and DIEA were distilled over calcium hydride. All reactions were performed under anhydrous conditions under argon. NMR experiments were accomplished on Bruker DRX 400 and AM 300 spectrometers at 20 °C. HRMS analyses were performed with electrospray ionization (ESI) in positive or negative mode on a Q-TOF Micromass spectrometer. Analytical and semi-preparative HPLC were performed on a Dionex DX 600 HPLC system equipped with anion-exchange DNAPac PA 100 columns (4 × 250 mm for analysis or 9 × 250 mm, Dionex). The following HPLC solvent systems were used: 20% CH_3CN in 25 mM Tris-HCl buffer, pH 8 (buffer A) and 20% CH_3CN containing 200 mM $NaClO_4$ in 25 mM Tris-HCl buffer, pH 8 (buffer B). Flow rates were 1.5 mL min^{-1} and 5 mL min^{-1} for analysis and semi-preparative purposes, respectively. MALDI-TOF mass spectra were recorded on a Voyager-DE spectrometer equipped with a N_2 laser (337 nm) (Perseptive Biosystems, USA) using 2,4,6-trihydroxyacetophenone as a saturated solution in a mixture of acetonitrile/0.1 M ammonium citrate solution (1 : 1, v/v) for the matrix. Analytical samples were mixed with the matrix in a 1 : 5 (v/v) ratio, crystallized on a 100-well stainless steel plate and analyzed. UV quantitation of RNAs was performed on a Varian Cary 300 Bio UV/Visible spectrometer by measuring absorbance at 260 nm.

2′-*O*-Acetylthiomethyl-3′-*O*-((2-cyanoethyl)(diisopropylamino)-phosphanyl)-5′-*O*-(4,4′-dimethoxytrityl) uridine (1)

To a solution of 2′-*O*-acetylthiomethyl-5′-*O*-(4,4′-dimethoxytrityl) uridine (4.28 g, 6.74 mmol, 1.00 equiv.) in anhydrous CH_2Cl_2 (52 mL) previously passed through an alumina column was added dropwise a mixture of *N*,*N*-diisopropylethylamine (2.94 mL, 16.85 mmol, 2.50 equiv.) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (3.31 mL, 14.83 mmol, 2.20 equiv.) in CH_2Cl_2. The mixture was stirred for 2.5 h at room temperature under argon. After reaction completion, ethyl acetate previously washed with a saturated aqueous $NaHCO_3$ solution was added and the reaction mixture was poured into a saturated NaCl/$NaHCO_3$ solution (1/1 v/v). The aqueous layer was extracted with ethyl acetate and organic layers were dried over Na_2SO_4. The solvent was concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with an isocratic elution of CH_2Cl_2 and acetone (9/1) containing 1% pyridine. The desired phosphoramidite **1** was obtained as white foam (5.05 g, 6.05 mmol, 90%). ^{31}P-NMR (121 MHz, CD_3CN): δ 150.0, 149.3. HRMS (ESI^+) *m*/*z* calcd for $C_{42}H_{51}N_4O_{10}PS$ $(M + H)^+$ 835.3142, found 835.3149.

2-(Benzyldisulfanyl)pyridine (2a)

To a stirred solution of 2,2′-dithiodipyridine (1.33 g, 6.04 mmol, 1.50 equiv.) in 1 : 1 MeOH/DMF (50 mL), was added dropwise a solution of benzyl mercaptan (0.50 g, 4.03 mmol, 1.00 equiv.) in 1 : 1 MeOH/DMF (8 mL). The yellow mixture was stirred under an argon atmosphere at room temperature for 30 minutes. Then the mixture was diluted with ethyl acetate (80 mL) and washed with saturated aqueous $NaHCO_3$ (3 × 40 mL) and brine (40 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with a mixture of cyclohexane and ethyl acetate (80 : 20). The desired compound **2a** was obtained as clear oil (759 mg, 3.25 mmol, 81%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.43 (dq, J = 4.8 Hz, J = 2.4 Hz, 1H, H_{py}); 7.54–7.47 (m, 2H, H_{py}); 7.32–7.20 (m, 5H, Ar); 7.09 (ddd, J = 6.3 Hz, J = 4.8 Hz, J = 2.4 Hz, 1H, H_{py}); 4.02 (s, 2H, CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 160.0 (Cq); 149.3 (C_{py}); 136.9 (C_{py}); 136.5 (Cq); 129.3, 128.5, 127.6 (C_{Ar}); 120.5 (C_{py}); 119.6 (C_{py}); 43.7 (CH_2). HRMS (ESI^+) *m*/*z* calcd for $C_{12}H_{11}NS_2$ $(M + H)^+$ 234.0411, found 234.0410

2-(Phenylethyldisulfanyl)pyridine (2b)

Using the same procedure as for the synthesis of **2a**, starting from phenylethylmercaptan (0.50 g, 3.62 mmol) compound **2b** was obtained as yellow oil (608 mg, 2.46 mmol, 68%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.48 (dq, J = 4.8 Hz, J = 1.2 Hz, 1H, H_{py}); 7.70–7.59 (m, 2H, H_{py}); 7.32–7.17 (m, 5H, Ar); 7.09 (ddd, J = 6.9 Hz, J = 4.8 Hz, J = 1.2 Hz, 1H, H_{py}); 3.08–2.99 (m, 4H, CH_2). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 160.3 (Cq); 149.5 (C_{py}); 139.6 (Cq); 137.2 (C_{py}); 128.6, 128.5, 126.5 (C_{arom}); 120.6 (C_{py}); 119.7 (C_{py}); 40.0 (CH_2); 35.3 (CH_2). HRMS (ESI^+) *m*/*z* calcd for $C_{13}H_{13}NS_2$ $(M + H)^+$ 248.0568, found 248.0571.

2-(Pyridin-2-yldisulfanyl)ethan-1-ol (2c)

To a stirred solution of 2,2′-dithiodipyridine (2.11 g, 9.60 mmol, 1.50 equiv.) in MeOH (50 mL), was added dropwise a solution of 2-mercaptoethanol (0.50 g, 6.40 mmol, 1.00 equiv.) in MeOH (12 mL). The yellow mixture was stirred under an argon atmosphere at room temperature for 30 minutes. The mixture was concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with a mixture of cyclohexane and ethyl acetate (60 : 40). The desired compound **2c** was obtained as a yellow solid (923 mg, 4.94 mmol, 77%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.47 (d, J = 4.8 Hz, 1H, H_{py}); 7.56 (t, J = 8.0 Hz, 1H, H_{py}); 7.39 (d, J = 8.4 Hz, 1H, H_{py}); 7.12 (dd, J = 7.2 Hz, J = 5.2 Hz, 1H, H_{py}); 3.78 (t, J = 5.2 Hz, 2H, CH_2); 2.93 (t, J = 5.2 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 159.0 (Cq); 149.7 (C_{py}); 136.8 (C_{py}); 121.8 (C_{py}); 121.4 (C_{py}); 58.2 (CH_2); 42.6 (CH_2). HRMS (ESI^+) *m*/*z* calcd for $C_7H_9NOS_2$ $(M + H)^+$ 188.0204, found 188.0205.

Ethyldisulfanyl-pyridine (2d)

Using the same procedure as for the synthesis of **2c**, starting from ethanethiol (0.50 g, 8.05 mmol) compound **2d** was obtained as yellow oil (835 mg, 4.88 mmol, 61%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.42 (dq, J = 4.8 Hz, J = 0.8 Hz, 1H, H_{py}); 7.69 (dt, J = 8.0 Hz, J = 0.8 Hz, 1H, H_{py}); 7.60 (td, J = 8.0 Hz, J = 2.0 Hz, 1H, H_{py}); 7.04 (ddd, J = 7.6 Hz, J = 4.8 Hz, J = 0.8 Hz, 1H, H_{py}); 2.77 (q, J = 7.6 Hz, 2H, CH_2); 1.30 (t, J = 7.6 Hz, 3H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 160.5 (Cq); 149.4 (C_{py}); 136.9 (C_{py}); 120.4(C_{py}); 119.4 (C_{py}); 32.7 (CH_2); 14.1 (CH_3).

HRMS (ESI$^+$) m/z calcd for $C_7H_9NS_2$ $(M + H)^+$ 172.0255, found 172.0257.

3-(Pyridin-2-yldisulfanyl)propionic acid (2e)

Using the same procedure as for the synthesis of **2c**, starting from 3-mercaptopropionic acid (0.50 g, 4.71 mmol) compound **2e** was obtained as a yellow solid (570 mg, 2.65 mmol, 56%). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 8.45 (dq, J = 4.8 Hz, J = 1.2 Hz, 1H, H$_{py}$); 7.81 (td, J = 8.0 Hz, J = 1.6 Hz, 1H, H$_{py}$); 7.75 (dt, J = 8.0 Hz, J = 1.2 Hz, 1H, H$_{py}$); 7.23 (ddd, J = 7.2 Hz, J = 4.8 Hz, J = 1.2 Hz, 1H, H$_{py}$); 3.00 (t, J = 6.8 Hz, 2H, CH$_2$); 2.64 (t, J = 6.8 Hz, 2H, CH$_2$). ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ 172.5 (C═O); 159.0 (Cq); 149.6 (C$_{py}$); 137.8 (C$_{py}$); 121.2 (C$_{py}$); 119.3 (C$_{py}$); 33.4 (CH$_2$); 33.3 (CH$_2$). HRMS (ESI$^+$) m/z calcd for $C_8H_9NO_2S_2$ $(M - H)^-$ 213.9996, found 213.9997.

2,2,2,-Trifluoro-*N*-(2-(pyridin-2-yldisulfanyl)ethyl)acetamide (2f)

To a stirred solution of cysteamine (0.50 g, 4.40 mmol, 1.00 equiv.) in methanol (3 mL) under argon, was added triethylamine (594 μL, 4.40 mmol, 1.00 equiv.) and ethyl trifluoroacetate (597 μL, 5.50 mmol, 1.25 equiv.). The mixture was stirred at room temperature for 3.5 h and then evaporated under pressure. The residue was dissolved in 1 : 1 MeOH/DMF (8 mL) and a solution of 2,2′-dithiodipyridine (1.46 g, 6.60 mmol, 1.50 equiv.) in 1 : 1 MeOH/DMF (50 mL) was added dropwise. The yellow mixture was stirred under an argon atmosphere at room temperature for 2 h. Then the mixture was diluted with ethyl acetate (80 mL) and washed with saturated aqueous $NaHCO_3$ (3 × 40 mL) and brine (40 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography with dichloromethane. The desired compound **2f** was obtained as a yellow solid (951 mg, 3.37 mmol, 77%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.61 (s, 1H, NH); 8.47 (d, J = 4.4 Hz, 1H, H$_{py}$); 7.60 (td, J = 8.0 Hz, J = 1.2 Hz, 1H, H$_{py}$); 7.40 (dt, J = 8.0 Hz, J = 1.2 Hz, 1H, H$_{py}$); 7.19 (ddd, J = 8.0 Hz, J = 4.4 Hz, J = 1.2 Hz, 1H, H$_{py}$); 3.67 (q, J = 6.0 Hz, 2H, CH$_2$); 2.93 (m, 2H, CH$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 158.6 (Cq); 157.3 (q, C═O); 149.7 (C$_{py}$); 137.0 (C$_{py}$); 122.3 (C$_{py}$); 121.9 (C$_{py}$); 117.0 (q, CF_3); 37.9 (CH$_2$); 36.6 (CH$_2$). ^{19}F-NMR (160 MHz, $CDCl_3$) δ −75.91. HRMS (ESI$^+$) m/z calcd for $C_9H_9N_2OF_3S_2$ $(M + H)^+$ 283.0187, found 283.0189.

S-(Pyridin-2-ylthio)-*N*-(2,2,2-trifluoroacetyl)cysteine (2g)

To a stirred solution of L-cysteine (250 mg, 2.06 mmol, 1.00 equiv.) in methanol (2 mL) under argon were added triethylamine (278 μL, 2.06 mmol, 1.00 equiv.) and ethyl trifluoroacetate (280 μL, 2.57 mmol, 1.25 equiv.). The mixture was stirred at room temperature overnight and then evaporated under pressure. The residue was dissolved in MeOH (4 mL) and a solution of 2,2′-dithiodipyridine (520 mg, 2.37 mmol, 1.15 equiv.) in MeOH (12 mL) was added dropwise. After stirring for 30 minutes, the yellow mixture was evaporated under reduced pressure. The crude material was purified by silica gel column chromatography with a step gradient of dichloromethane and methanol (5–8%). The desired compound **2g** was obtained as a pale brown solid (287 mg, 0.88 mmol, 43%). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ 9.90 (d, J = 7.6 Hz, 1H, NH); 8.46 (dq, J = 4.8 Hz, J = 1.2 Hz, 1H, H$_{py}$); 7.79 (td, J = 8.0 Hz, J = 1.6 Hz, 1H, H$_{py}$); 7.71 (dt, J = 8.0 Hz, J = 1.2 Hz, 1H, H$_{py}$); 7.26 (ddd, J = 7.6 Hz, J = 4.8 Hz, J = 1.2 Hz, 1H, H$_{py}$); 4.55 (m, 1H, CH); 3.35 (dd, J = 14.0 Hz, J = 4.0 Hz, 1H, CH$_2$); 3.21 (dd, J = 14.0 Hz, J = 10.4 Hz, 1H, CH$_2$). ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ 170.1 (C═O); 158.2 (Cq); 156.5 (q, C═O); 149.7 (C$_{py}$); 137.7 (C$_{py}$); 121.4 (C$_{py}$); 119.5 (C$_{py}$); 117.0 (q, CF_3); 51.9 (CH); 38.6 (CH$_2$). ^{19}F-NMR (160 MHz, DMSO-d$_6$) δ −74.32. HRMS (ESI$^+$) m/z calcd for $C_{10}H_9N_2O_3F_3S_2$ $(M + H)^+$ 327.0085, Found 327.0086.

Synthesis of partially modified 2′-*O*-AcSM oligonucleotides

Oligonucleotides were synthesized on an ABI model 394 DNA/RNA synthesizer on a 1 μmol scale using commercially available 2′-*O*-PivOM phosphoramidites (Chemgenes), 2′-*O*-AcSM uridine phosphoramidite **1** and a controlled-pore glass (CPG) commercial solid support. Oligonucleotides were assembled in TWIST™ synthesis columns (Glen Research). Phosphoramidites were vacuum dried prior to their dissolution in extra dry acetonitrile (Biosolve) at 0.1 M concentration. Coupling for 180 s was performed with 5-benzylmercaptotetrazole (BMT, 0.3 M) as an activator. The oxidizing solution was 0.1 M iodine in THF/pyridine/H_2O (78 : 20 : 2; v/v/v) (Link Technologies). The capping step was performed with a mixture of 5% phenoxyacetic anhydride (Pac_2O) in THF and 10% *N*-methylimidazole in THF (Link Technologies). Detritylation was performed with 3% TCA in CH_2Cl_2. After RNA assembly completion, the column was removed from the synthesizer and dried under a stream of argon.

Synthesis of partially modified 2′-*O*-RSSM oligonucleotides (4a–g)

1 mL of a 0.4 M alkyldisulfanyl-pyridine **2a–g** (400 equiv.) solution and a 2.5 M butylamine (250 μL) solution in anhydrous THF (750 μL) was applied to the column containing the 2′-*O*-AcSM oligonucleotide for 15 min using two glass syringes filled with 4 Å molecular sieves (5 beads each). Then the solution was removed and the solid-support was washed with anhydrous THF followed by a 1 min flush with argon. The solid support was treated with a 30% aqueous ammonia solution for 3 h. The deprotection solution was evaporated in the presence of isopropylamine (13% of total volume) under reduced pressure. The crude oligonucleotide was diluted in water, transferred in a 2 mL Eppendorf vial and then lyophilized from water.

Analysis, purification and desalting of 2′-*O*-RSSM oligonucleotides (4a–g)

The crude 2′-*O*-RSSM oligonucleotides **4a–g** were analyzed by anion-exchange HPLC using a specific linear gradient of buffer B in buffer A for each RNA, and they were characterized by MALDI-TOF spectrometry. The crude mixtures were then purified by semi-preparative IEX-HPLC with a specific linear gradient of buffer B in buffer A. The pure fractions of RNA were

pooled in a 100 mL round-bottomed flask and were concentrated to dryness under reduced pressure. The residues were dissolved in 100 mM TEAAc buffer, pH 7 (8 mL divided into three portions for rinsing the flask: 5 mL, 2 mL, and 1 mL) and were loaded on a C_{18} cartridge (Waters, Sep-Pak®). Elution was performed with 10 mL of a 100 mM TEAAc solution then with a 10 mL of 50% CH_3CN in 12.5 mM TEAAc solution. The second fraction containing the desired compound was collected in a 100 mL round-bottomed flask and was freeze-dried. The residue was dissolved in 1.5 mL water (divided into 3 portions of 0.8 mL, 0.4 mL, and 0.3 mL for rinsing the flask) and transferred to a 2 mL Eppendorf-vial and lyophilized from water. Purified oligonucleotides **4a–g** were analyzed by MALDI-TOF analysis and quantified by UV measurement. Lyophilized RNAs were stored at −20 °C for several months without any degradation.

Thermal denaturation experiments

T_m experiments were performed on a CARY 300 UV Spectrophotometer (Varian Inc.) equipped with a Peltier temperature controller and thermal analysis software. The samples were prepared by mixing oligonucleotide solutions at 3 μM final concentration in 10 mM sodium cacodylate, 100 mM NaCl, pH 7. A heating–cooling–heating cycle in the 5–90 °C temperature range with a gradient of 0.5 °C min^{-1} was applied. T_m values were determined from the maxima of the first derivative plots of absorbance *versus* temperature.

CD spectroscopy studies

CD spectra were recorded on a Jasco J-815 spectropolarimeter. Non-modified and modified duplexes were diluted to a concentration of 3 μM in a 10 mM sodium cacodylate buffer and 100 mM sodium chloride, pH 7 in a total volume of 1 mL. Measurements were performed in a 1 cm path length quartz cuvette at 1 °C. The wavelength range was set to 340–200 nm with a scanning speed of 100 nm min^{-1}. Raw data were acquired over 3 scans.

Evaluation of resistance of 2′-*O*-RSSM oligonucleotides to SVPDE

2′-*O*-RSSM oligonucleotides **4a–g** (500 pmol) were incubated with a mixture of 2 μL of SVPDE ($C_{initial}$ = 0.05 mU μL^{-1}), 2 μL of citrate ammonium solution (50 g L^{-1}) and 14 μL of distilled water at 37 °C. At different times of incubation, 2 μL were withdrawn and mixed with a solution of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA) (10/1 v/v) ($C_{initial\ of\ HPA\ and\ PA}$ = 50 g L^{-1}). This mixture was crystallized on a 100-well stainless steel plate and analyzed by MALDI-TOF MS.

Conversion of 2′-*O*-RSSM oligonucleotides into 2′-OH oligonucleotides under glutathione treatment

Duplexes formed with 2′-*O*-RSSM oligonucleotides **4a–g** (56 μM) were mixed with a solution of glutathione in an HEPES buffer (34 mM NaCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 7.5 mM HEPES, pH 8) (glutathione final concentration 5.6 mM). After 1 h incubation at 37 °C, the samples were analyzed by IEX-HPLC and MALDI-TOF MS.

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Notes and references

1 A. Wittrup and J. Lieberman, *Nat. Rev. Genet.*, 2015, **16**, 543–552.
2 J. K. W. Lam, M. Y. T. Chow, Y. Zhang and S. W. S. Leung, *Mol. Ther. – Nucleic Acids*, 2015, **4**, e252.
3 G. F. Deleavey and M. J. Damha, *Chem. Biol.*, 2012, **19**, 937–954.
4 S. Shukla, C. S. Sumaria and P. I. Pradeepkumar, *ChemMedChem*, 2010, **5**, 328–349.
5 A. Biscans, S. Rouanet, J.-R. Bertrand, J.-J. Vasseur, C. Dupouy and F. Debart, *Bioorg. Med. Chem.*, 2015, **23**, 5360–5368.
6 A. Biscans, M. Bos, A. R. Martin, N. Ader, G. Sczakiel, J.-J. Vasseur, C. Dupouy and F. Debart, *ChemBioChem*, 2014, **15**, 2674–2679.
7 T. Lavergne, C. Baraguey, C. Dupouy, N. Parey, W. Wuensche, G. Sczakiel, J.-J. Vasseur and F. Debart, *J. Org. Chem.*, 2011, **76**, 5719–5731.
8 N. Parey, C. Baraguey, J.-J. Vasseur and F. Debart, *Org. Lett.*, 2006, **8**, 3869–3872.
9 G. Tosquellas, K. Alvarez, C. Dell'Aquila, F. Morvan, J.-J. Vasseur, J.-L. Imbach and B. Rayner, *Nucleic Acids Res.*, 1998, **26**, 2069–2074.
10 B. R. Meade, K. Gogoi, A. S. Hamil, C. Palm-Apergi, A. Van Der Berg, J. C. Hagopian, A. D. Springer, A. Eguchi, A. D. Kacsinta, C. F. Dowdy, A. Presente, P. Lönn, M. Kaulich, N. Yoshioka, E. Gros, X. S. Cui and S. F. Dowdy, *Nat. Biotechnol.*, 2014, **32**, 1256–1261.
11 C. Ducho, *ChemMedChem*, 2015, **10**, 1625–1627.
12 Q. Zhang, J. He, M. Zhang and P. Ni, *J. Mater. Chem. B*, 2015, **3**, 4922–4932.
13 Z. Xu, S. Liu, Y. Kang and M. Wang, *Nanoscale*, 2015, **7**, 5859–5868.
14 M. Gund, A. Khanna, N. Dubash, A. Damre, K. S. Singh and A. Satyam, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 122–127.
15 A. El Alaoui, F. Schmidt, M. Amessou, M. Sarr, D. Decaudin, J.-C. Florent and L. Johannes, *Angew. Chem., Int. Ed.*, 2007, **46**, 6469–6472.
16 Y. Guo, H. Wang, Y. Su and B. Qu, *Chem. Commun.*, 2012, **48**, 3221–3223.
17 R. Hong, G. Han, J. M. Fernandez, B. J. Kim, N. S. Forbes and V. M. Rotello, *J. Am. Chem. Soc.*, 2006, **128**, 1078–1079.
18 A. Semenyuk, A. Földesi, T. Johansson, C. Estmer-Nilsson, P. Blomgren, M. Brännvall, L. A. Kirsebom and M. Kwiatkowski, *J. Am. Chem. Soc.*, 2006, **128**, 12356–12357.

19 T. A. Lönnberg, *ChemBioChem*, 2012, **13**, 2690–2700.
20 A. Semenyuk and M. Kwiatkowski, *Tetrahedron Lett.*, 2007, **48**, 469–472.
21 Y. Ochi, O. Nakagawa, K. Sakaguchi, S. I. Wada and H. Urata, *Chem. Commun.*, 2013, **49**, 7620–7622.
22 K. Mori, T. Kodama, T. Baba and S. Obika, *Org. Biomol. Chem.*, 2011, **9**, 5272–5279.
23 V. Metelev, A. Romanenkov, E. Kubareva, E. Zubin, N. Polouchine, T. Zatsepin, N. Molochkov and T. Oretskaya, *IUBMB Life*, 2006, **58**, 654–658.
24 T. Lavergne, J.-R. Bertrand, J.-J. Vasseur and F. Debart, *Chem. – Eur. J.*, 2008, **14**, 9135–9138.
25 A. B. Smith, S. N. Savinov, U. V. Manjappara and I. M. Chaiken, *Org. Lett.*, 2002, **4**, 4041–4044.
26 J. V. Castell and A. Tun-Kyi, *Helv. Chim. Acta*, 1979, **62**, 2507–2510.
27 I. P. Smirnov, M. T. Roskey, P. Juhasz, E. J. Takach, S. A. Martin and L. A. Haff, *Anal. Biochem.*, 1996, **238**, 19–25.
28 A. De Mesmaeker, R. Häner, P. Martin and H. E. Moser, *Acc. Chem. Res.*, 1995, **28**, 366–374.
29 M. Teplova, S. T. Wallace, V. Tereshko, G. Minasov, A. M. Symons, P. Dan Cook, M. Manoharan and M. Egli, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 14240–14245.
30 R. H. Griffey, B. P. Monia, L. L. Cummins, S. Freier, M. J. Greig, C. J. Guinosso, E. Lesnik, S. M. Manalili, V. Mohan, S. Owens, B. R. Ross, H. Sasmor, E. Wancewicz, K. Weiler, P. D. Wheeler and D. Cook, *J. Med. Chem.*, 1996, **39**, 5100–5109.