



HAL
open science

Conjugation of Small Molecules to RNA Using a Reducible Disulfide Linker Attached at the 2'-OH Position through a Carbamate Function

Florian Gauthier, Astrid Malher, Jean-Jacques Vasseur, Christelle Dupouy, Françoise Debart

► **To cite this version:**

Florian Gauthier, Astrid Malher, Jean-Jacques Vasseur, Christelle Dupouy, Françoise Debart. Conjugation of Small Molecules to RNA Using a Reducible Disulfide Linker Attached at the 2'-OH Position through a Carbamate Function. *European Journal of Organic Chemistry*, 2019, 2019 (33), pp.5636-5645. 10.1002/ejoc.201900740 . hal-02307249

HAL Id: hal-02307249

<https://hal.science/hal-02307249>

Submitted on 6 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Conjugation of small molecules to RNA using a reducible disulfide linker attached at 2'-OH position *via* a carbamate function

Florian Gauthier,^[a] Astrid Malher,^[a] Jean-Jacques Vasseur,^[a] Christelle Dupouy,^{*[a]} and Françoise Debart ^{*[a]}

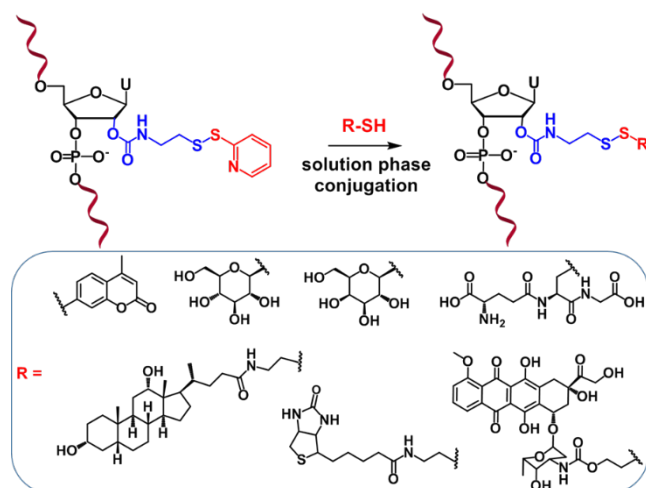
Abstract: A post-synthesis conjugation method was developed to functionalize oligoribonucleotides with various small molecules of biological interest using a reduction-sensitive disulfide linker connected to 2'OH via a carbamate function. For this, first we prepared a 2'-O-[(*N*-(acetylthio)-ethylcarbamoyl)] (2'-O-AcSEC) uridine phosphoramidite as the key precursor of the disulfide linker, that was incorporated into 21-mer RNAs. Next the 2'-O-AcSEC group was converted on solid support to the activated 2'-O-[*N*-(2-pyridyldisulfanyl)-ethylcarbamoyl] (2'-O-PySSEC) group. Finally, the coupling between 2'-O-PySSEC-modified RNA and diverse small molecules bearing a thiol function was based on a thiol-disulfide exchange reaction in solution. Several RNA-small molecule conjugates were obtained with satisfactory conjugation yields. An RNA was successfully double-conjugated with an anticancer drug (doxorubicin) attesting the efficiency and the robustness of the conjugation method. We have shown that the disulfide-linked RNA-small molecule conjugates were cleaved upon 5.6 mM glutathione treatment, featuring the release of the small molecules in cells.

Introduction

Conjugation of oligonucleotides (ON) with small molecules is challenging and arouses researcher's interest for diverse applications such as therapeutics or diagnosis. Covalent association to small molecules such as peptides, carbohydrates or lipids could improve ON properties such as cell-specific delivery, cellular uptake efficiency or half-lives in fluids.^[1] Carbohydrates are recognized by protein receptors named lectins expressed on cell surfaces. As glycoproteins containing sugar moieties are internalized into cells, ON-carbohydrate conjugates can therefore be used to improve poor cell or tissue-specific uptake of ON.^[2] In particular, *N*-acetylgalactosamine (GalNAc) siRNA conjugates should be mentioned as one significant and relevant example of the successful use of sugar-oligonucleotide

conjugates for delivery to liver hepatocytes.^[3] Conjugation of small molecules to ON could also be used for detection. Recently, coumarin derivative attached to ON was used as a fluorescent probe to specifically detect glutathione, the most abundant biothiol over cysteine and homocysteine.^[4] Moreover, vitamins receptors, such as biotin receptors, are overexpressed in many cancers and consequently were used as biomarkers for the imaging and the detection of tumors cells or the targeting of drug delivery.^[5]

Attachment of small molecules to ON using various chemical strategies with amide^[6] or oxime^[7] linkers or *via* click chemistry^[8] has been reported. Due to its high compatibility with many functional groups and the ease of formation, the disulfide bond is also very attractive for conjugation. Indeed, this linkage is stable under physiological conditions and is cleaved in the intracellular reducing environment.^[9] The conjugation reactions using a disulfide linkage were mostly achieved at the 5'- and 3'-end positions of ON due to their great accessibility.^[10] Likewise, the nucleobases^[11] and the phosphodiester bonds^[12] were selected as other conjugation sites. However, these positions suffer from different various limitations: only one molecule can be conjugated to ON at 5'- or 3'-end, the base-pairing can be disturbed by conjugation on the nucleobases and RNA structure can be altered by modifications of the phosphates. In contrast, the 2'-position is more versatile and allows multi-conjugation sites within one sequence. To our knowledge, only a few examples of conjugation to 2'-position of ribonucleosides *via* a disulfide linkage have been reported. Winkler and coll. developed a solid-phase coupling of peptides and polyethylene glycols ligands to ON *via* a 2'-O-thioethyl nucleoside attached to the solid support through the thiol function.^[13]



[a] IBMM, University of Montpellier, CNRS, ENSCM, Montpellier, France

E-mail: christelle.dupouy@umontpellier.fr
francoise.debart@umontpellier.fr

FULL PAPER

Scheme 1 Synthesis of 2'-O-[N-(2-ethylthio)ethyl carbamoyl] RNA-small-molecule conjugates.

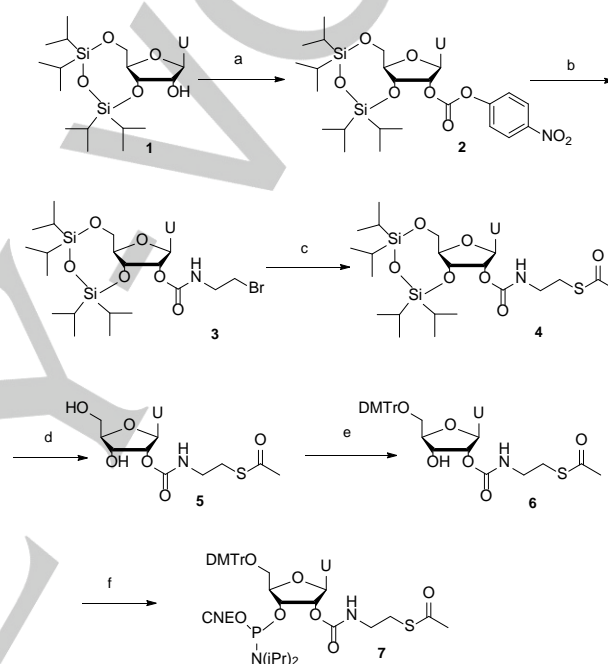
This method is efficient yet only the 3'-extremity of ON can be conjugated. Another example of conjugation reaction at the 2'-position with a disulfide linker has been reported for the specific cross-linking of proteins *via* cysteine to ON. In this case, a 2'-amino-2'-deoxyuridine was introduced as a precursor unit within the ON sequence then the amino group was converted to 2'-pyridyldithiopropionamide derivative to perform the thiol-disulfide exchange reaction with cysteine yielding the disulfide linker.^[14] Moreover, in a previous work we developed a post-synthesis strategy based on the thiol-disulfide exchange reaction for the preparation of siRNA prodrugs 2'-O-modified by disulfide-containing groups.^[15] We also used this reaction to introduce a disulfide-bridge between 2'-O-positions of two adjacent nucleotides in the loop of RNA hairpins.^[16] In both cases, the disulfide bond was anchored to the nucleoside *via* a thioacetal function. Reduction of the disulfide bond resulted in an unstable hemithioacetal giving rise to free 2'-OH RNA.

In this paper, we present a post-synthesis conjugation method of a 21-mer RNA to various small molecules of interest such as carbohydrates, lipids, vitamins, fluorescent probes and anticancer drugs using a disulfide linker attached at 2'-position *via* a carbamate function (Scheme 1). It is well known that carbamate function plays an important role in medicinal chemistry due to its high stability in physiological conditions.^[17] Moreover, several functional groups (dansyl, amino aliphatic chains) were previously introduced into the 2'-OH position of ON *via* a carbamate function.^[18] 2'-carbamate modification is stable to conditions of standard oligonucleotide synthesis using phosphoramidite chemistry. The 2'-carbamate functionalization of RNA with various biomolecules for many applications, *via* a reduction-responsive disulfide linker remained quite unexplored. The global strategy for the synthesis of these RNA-small molecule conjugates is based on a thiol-disulfide exchange reaction between a small molecule containing a thiol or a thiolate function and an RNA bearing a pyridyldisulfanyl group (Scheme 1). The driving force of this reaction is the formation and elimination of the pyridine-2-thione, which allows a rapid and irreversible reaction.

Results and Discussion

Synthesis of 2'-O-AcSEC uridine phosphoramidite 7. To anchor the linker at 2'-position of RNA, we first prepared 2'-O-[(N-(acetylthio)-ethylcarbamoyl] (2'-O-AcSEC) uridine 3'-phosphoramidite **7** which represents the precursor unit for 2'-functionalization within RNA sequence (Scheme 2). The 2'-carbamate modification was achieved *via* a 2'-O-carbonate intermediate **2** obtained with 81% yield by treating 3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl) (TIPS) uridine **1** with 4-nitrophenylchloroformate.^[18b] The 2'-O-[N-ethylbromide carbamoyl] uridine **3** was formed with 2-bromoethylamine hydrobromide in 98% yield. Then, a nucleophilic substitution of

bromine by potassium thioacetate in the presence of 18-crown-6 gave 2'-O-AcSEC uridine **4** in 80% yield. A treatment with Et₃N·3HF yielded the 3',5'-OH 2'-O-AcSEC uridine **5** (92% yield), which was further selectively protected at 5'-O-position with a dimethoxytrityl group to give the nucleoside **6** (98% yield). Finally, phosphitylation of **6** with chloro-(2-cyanoethoxy)-(N,N-diisopropylamino)phosphine afforded the 3'-phosphoramidite 2'-O-AcSEC uridine **7** with high purity in 89% yield. The synthesis of 2'-O-AcSEC uridine phosphoramidite **7** was efficiently achieved in 6 steps with 51% overall yield from 5',3'-O-TIPS-uridine **1**.

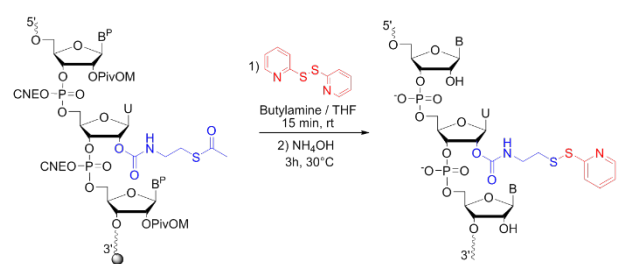


Scheme 2. Synthesis of 2'-O-AcSEC uridine phosphoramidite **7**. Reagent and conditions: a) 4-nitrophenyl chloroformate, pyridine, toluene, r.t., 5 h, 81%; b) 2-bromoethylamine hydrobromide, Et₃N, pyridine, r.t., 4 h, 98%; c) CH₃COSK, 18-crown-6, CH₃CN, r.t., overnight, 80%; d) Et₃N·3HF, THF, r.t., 4 h, 92%; e) DMTrCl, DIEA, pyridine, CH₂Cl₂, r.t., 7 h, 98%; f) iPr₂NPCl(OCNE), DIEA, CH₂Cl₂, r.t., 3.5 h, 89%

Synthesis of 21-mer RNAs with one or two 2'-O-PySSEC modifications. The 2'-O-AcSEC uridine phosphoramidite **7** was incorporated once or twice into 21-mer RNA whose sequence corresponds to the sense strand of a siRNA (**siEF-4S**) targeting a gene involved in Ewing's sarcoma. The 2'-O-modification was introduced either in the middle of the sequence (RNA **8**) or at two sites: in the middle and close to the 3'-end (RNA **9**) (Table 1). Both RNAs were synthesized using the phosphoramidite uridine **7** and commercially available 2'-O-pivaloyloxymethyl (2'-O-PivOM) ribonucleoside phosphoramidites (Chemgenes) as precursors of 2'-OH ribonucleotides.^[19] From the same 2'-O-AcSEC RNA precursor, various 2'-O-functionalized RNAs with different molecules bearing a thiol function can be prepared. The syntheses were carried out on a 1 μmol scale with a 180 s

coupling step. After RNA assembly, an activation step of the thiol function into pyridyldisulfanyl ethylcarbamate (2'-O-PySSEC) was performed on solid support, by treating 2'-O-AcSEC RNAs with a large excess of 2,2'-dithiodipyridine (1000 equiv. per modification) in anhydrous BuNH₂/THF (1/3) solution for 15 min. During this reaction, cyanoethyl protecting groups were also removed from phosphates. Then 2'-O-PySSEC RNAs were released from solid support and deprotected (nucleobases and 2'-OH) with an aqueous concentrated ammonia solution for 3 h at 30°C. Crude 2'-O-PySSEC RNAs **8** and **9** were purified by Ion-Exchange HPLC (IEX-HPLC), characterized by MALDI-TOF mass spectrometry and obtained with high purity (Figures S10-S11, Supplementary Information).

Table 1. Synthesis of 2'-O-PySSEC RNAs **8** and **9**



B^P = N⁴-acetyl cytosine (a) ; N⁶-Pac adenine (b) ; N²-Pac guanine (c) ; uracile (d). Pac : phenoxyacetyl group

Figure 1. Structures of small molecules used for conjugation to RNA

RNA	Sequence [a]	MALDI-TOF MS [b]	
		Calcd.	Found
siEF-4S	5'GCAGCAGAACCCUUCUUAUGA 3'	/	/
8	5'GCAGCAGAACCCU _R UCUUAUGA 3'	6867.4	6867.9
9	5'GCAGCAGAACCCU _R UCUUAU _R GA 3'	7079.7	7079.5

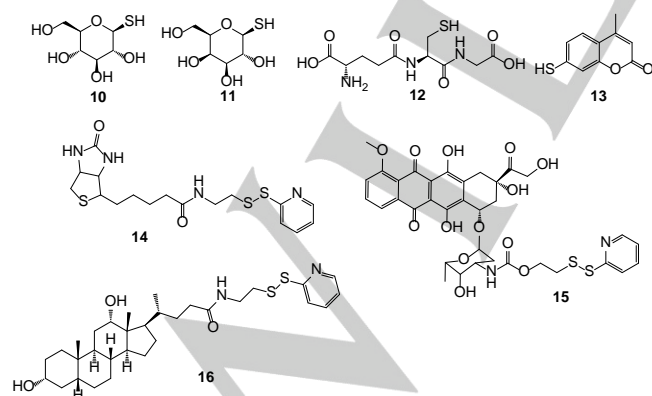
[a] U_R = 2'-O-PySSEC Uridine. [b] Negative mode

Conjugation of 2'-O-PySSEC RNA to various small molecules.

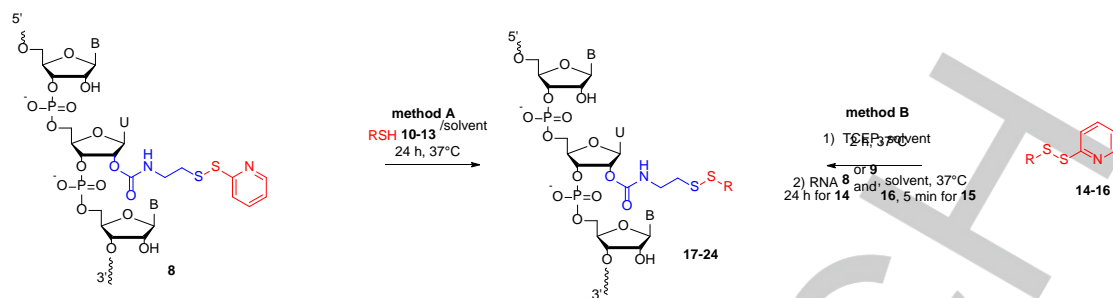
A large variety of molecules such as galactose, glucose, glutathione deoxycholic acid, biotin, 4-methylcoumarin and doxorubicin (Dox) have been anchored to a 21-mer RNA (Scheme 1). Moreover, this RNA has been functionalized at one or two separate sites with Dox, an anthracycline cytotoxic chemotherapeutic drug employed for the treatment of several cancers such as breast, stomach, soft tissue sarcomas and lymphomas cancers. 2'-O-PySSEC RNA **8** was conjugated in solution to small molecules **10-16** bearing thiol or disulfide functions using a thiol-disulfide exchange reaction (Figure 1, Table 2). For the coupling, two slightly different methods A and B were investigated depending on the commercial availability of the thiol derivative for each small molecule.

Method A corresponds to the direct coupling of commercially available thiol compounds such as 1-thio-β-D-glucose **10**, 1-thio-β-D-galactose **11**, glutathione **12** and 7-mercapto-4-methylcoumarin **13** to 2'-O-PySSEC RNA **8**. The reactions were performed in HEPES buffer (pH = 8) at 37°C for 24 h except for coumarin derivative **13** where DMF (50%) was added to improve solubility (Table 2). After coupling completion, the resulting conjugates were purified by IEX-HPLC and characterized by MALDI-TOF mass spectrometry (Table 2, Figures S12-S15). For example, Figure 2a shows the HPLC chromatograms of the crude conjugates resulting from the coupling between RNA **8** with 1-thio-β-D-glucose **10** (left panel) or glutathione **12** (right panel), respectively. In both cases, the major peak representing 63% and 70% was assigned to RNA conjugates **17** and **19** obtained with reasonable efficacy. RNA conjugates **18** and **20**, corresponding to the functionalization of RNA **8** with 1-thio-β-D-galactose **11** and 7-mercapto-4-methylcoumarin **13** respectively, were obtained with higher efficacy (85% and 72% calculated by integration of chromatogram at 260 nm) (Table 2).

However, after IEX-HPLC purification all conjugates were isolated with much lower recovery yields (from 36% for **17** to 46% for **18** and **20**). The additional desalting step performed after IEX-HPLC purification could explain the difference between the conjugation efficacies and the recovery yields. Moreover, in HPLC chromatograms, minor peaks with shorter retention times are noteworthy (Figure 2a, Figures S12-S15). These peaks were assigned to shorter RNA sequences corresponding to some chemical degradations of RNA probably due to the excess of small molecules at high concentration (100 mM) in the reaction mixture. To check this hypothesis, we monitored the stability of the unmodified RNA siEF-4S in the presence of small molecules **10-13** by IEX-HPLC (Figure S19). After 24 h incubation, the peaks corresponding to siEF-4S mostly decreased in the presence of 1-

Table 2: Synthesis and data for RNA-small molecules conjugates **17-24** linked by a disulfide linkage

thio-β-D-glucose **10** (33%), 1-thio-β-D-galactose **11** (54%), 7-mercapto-4-methylcoumarin **13** (6%), respectively whereas siEF-4S remained more stable (89 %) in the presence of glutathione **12**.



ON ^[a]	R	Method ^[b]	solvent	MALDI-TOF MS ^[c]		Conjugation Yield % ^[d]	Recovery Yield % ^[e]
				Calcd.	Found		
17		A	HEPES buffer pH 8	6952.4	6952.8	63	37
18		A	HEPES buffer pH 8	6952.4	6953.1	85	46
19		A	HEPES buffer pH 8	70662.6	7063.7	70	42
20		A	HEPES buffer pH 8/DMF 1/1	6948.4	6945.4	72	46
21		B	HEPES buffer pH 8/DMF 3/1	7059.6	7061.8	89	46
22		B	HEPES buffer pH 8/DMF 1/1	7403.9	7405.9	88	63
23		B	HEPES buffer pH 8/DMF 7/3	7207.9	7207.5	42	36
24		B	HEPES buffer pH 8/DMF 1/1	8152.6	8152.9	71	38

[a] RNA sequence = $5^{\prime}\text{GCAGCAGAACCCU}_R\text{UCUUAUGA}^3$ for RNA-conjugates **17-23** ; RNA sequence = $5^{\prime}\text{GCAGCAGAACCCU}_R\text{UCUUAU}_R\text{GA}^3$ for RNA-Dox conjugate **24**, U_R = 2'-O-modified Uridine with R = small molecules. [b] Method A = RSH **10-13**/solvent, 37°C, 24 h ; Method B = 1) RSSPy **14-16** TCEP solvent 37°C, 2 h. 2) RNA **8** and **9** 37°C, 24 h for RNA conjugates **21** and **23**, 5 min for RNA conjugates **22** and **24**. [c] Negative mode. [d] percentage of peak area corresponding to the RNA conjugate in crude material as calculated by integration of the IEX-HPLC chromatogram monitored at 260 nm. [e] Recovery yield based on UV absorption at 260 nm of purified RNA conjugates **17-21** and **23** and at 481 nm for RNA conjugates **22** and **24**.

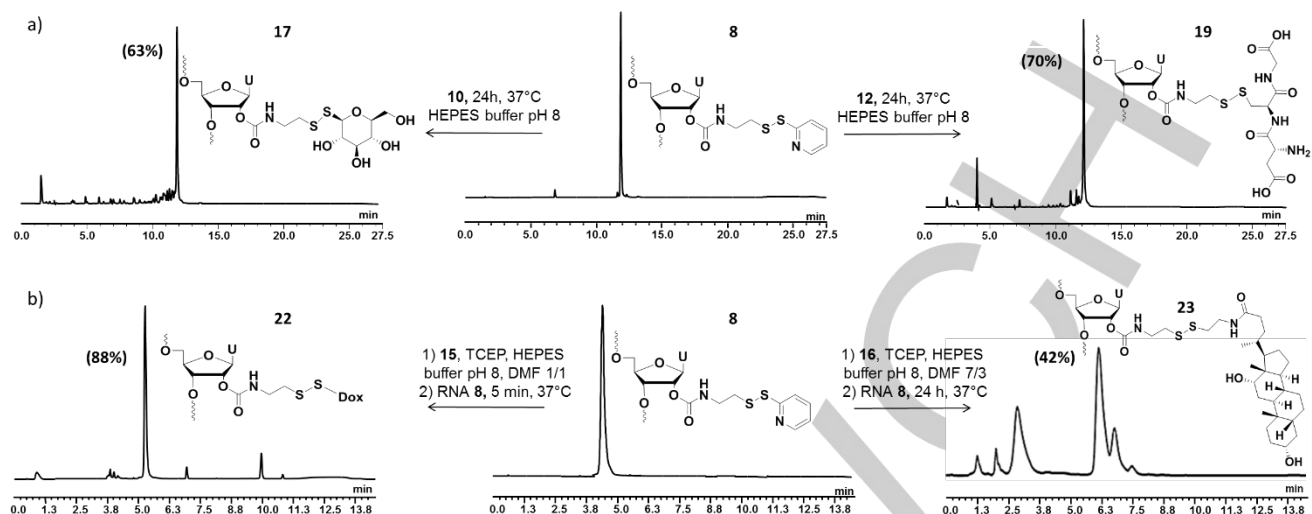


Figure 2. a) IEX-HPLC profiles of solution phase conjugation of RNA **8** with 1-thio- β -D-glucose **10** to give RNA-glucose conjugate **17** (left panel) and glutathione **12** to give RNA-glutathione conjugate **19** (right panel) upon method A. b) RP-HPLC profiles of solution phase conjugation of RNA **8** (top panel) with Dox-SS-py derivative **15** (left panel) and deoxycholic acid derivative **16** (right panel) upon method B to afford RNA conjugates **22** and **23**, respectively.

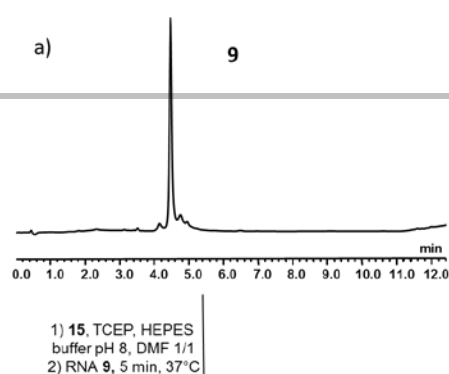
These results confirm that high concentration of 1-thio- β -D-glucose **10**, 1-thio- β -D-galactose **11** or 7-mercapto-4-methylcoumarin **13** causes some RNA degradation and might partially explain the low recovery yields.

The conjugation with more hydrophobic molecules was also evaluated using an alternative method B. In this case, the pyridyldisulfanyl derivatives of biotin and deoxycholic acid **14** and **16**, respectively, have been synthesized from biotin and deoxycholic acid that are not commercially available with a thiol function (Schemes S1-S2).^[20] Likewise, the doxorubicin pyridyldisulfanyl derivative (Dox-SS-Py) **15** was prepared following a described protocol.^[21] The coupling of the pyridyldisulfanyl derivatives **14**, **15** and **16** has been achieved upon method B (Table 2). First, they were treated with tris(2-carboxyethyl) phosphine (TCEP) in a mixture of HEPES buffer and DMF for 2 h to generate a thiol function. Then, this solution was directly added to the dry 2'-O-PySSEC RNA **8** upon stirring. The reaction was performed at 37°C for 24 h and monitored by IEX-HPLC for conjugate **21** (Figure S16) and RP-HPLC for **22** and **23** to optimize chromatographic separation (Figures 2b, S17-S18). After 24 h, the crude RNA conjugates were purified by IEX-HPLC or RP-HPLC and characterized by MALDI-TOF mass spectrometry. When treating RNA **8** with Dox-SS-Py **15**, some RNA degradations were observed within 24 h, the yield was improved by reducing the coupling time. Indeed, after 5 min incubation with Dox-SS-Py **15**, the peak corresponding to RNA **8** in the HPLC chromatogram has totally disappeared and the newly major peak was assigned to RNA conjugate **22** with satisfactory conjugation and recovery yields (88% and 63%, respectively) (Figure 2b left panel, Table 2). A similar estimated conjugation yield (89%) was obtained for RNA conjugate **21** with biotin after 24 h coupling time (Table 2). In contrast, RNA conjugate **23** with deoxycholic acid was formed with lower conjugation yields (42%) after 24 h reaction (Figure 2b, right panel). The steric hindrance of deoxycholic acid derivative could partially explain this low yield.

Moreover, supplementary peaks were also detectable in the HPLC chromatogram, probably due to the chemical degradation of RNA caused by high concentration of deoxycholic acid derivative **16**.

To check this hypothesis, the stability of unmodified RNA **siEF-4S** in the presence of pyridyldisulfanyl derivatives **14**, **15** and **16** was monitored by IEX-HPLC. Compounds **14**, **15** and **16** were treated with TCEP in a mixture of HEPES buffer and DMF for 2 h and were added to **siEF-4S**. After 24 h and 48 h incubation, **siEF-4S** remained nearly intact in the presence of **14**, **15** and **16** (Figure S20). Nevertheless, after 72 h, **siEF-4S** was slightly hydrolyzed in the presence of biotin derivative **14** (corresponding peak at 87%) and more significantly in the presence of doxorubicin (corresponding peak at 62%) whereas **siEF-4S** was totally degraded in the presence of deoxycholic acid derivative **16**. The steric hindrance of **16** combined to chemical degradation of RNA might explain the lower conjugation efficiency and recovery yield for RNA-deoxycholic acid conjugate **23**.

In order to synthesize a multi-conjugated RNA, we explored the conjugation reaction between Dox-SS-Py derivative **15** and RNA **9** bearing 2'-O-PySSEC modifications on two distinct ribonucleotides. Indeed, combination therapy of cancer using two drugs with different mechanisms of action (RNAi and chemotherapy with Dox) has aroused great interest for many years. Both drugs exhibit an individual biological activity in a synergistic manner in cancer cells and can overcome multidrug resistance, thereby inhibiting tumor progression. Up to now, numerous attempts have been made to co-deliver anticancer drugs and siRNA into cancer cells using a wide variety of nanocarriers (nanoparticles, micelles, liposomes).^[22]



peak corresponding to the initial RNA-biotin conjugate **21** (m/z 7061.8) has disappeared and the peak at m/z 6764.2 was assigned to the intermediate thiol resulting from the disulfide cleavage.

A new peak at m/z 7066.6 corresponding to the conjugate RNA-GSH **19** is noteworthy in the MS spectrum after 1.5 h incubation. Indeed, the released thiol has probably attacked the disulfide bond of the glutathione disulfide (GSSG), produced upon the spontaneous oxidation of GSH. After 6 h, RNA-GSH **19** was the sole compound detected in mass spectrometry and remained perfectly stable after 24 h. The same result was observed for all RNA-small molecule conjugates (Figures S21-S26). Moreover, after 6 h, mass spectra of RNA conjugates **17**, **20** and **23** showed important RNA degradations attesting the RNA sensitivity to 1-thio- β -D-glucose, 7-mercapto-4-methylcoumarin and deoxycholic acid derivatives (Figures S21, S24, S26). These results evidence that the disulfide bond is rapidly cleaved in the presence of GSH leading to a thiol derivative that is very reactive to conjugation with other disulfide-containing molecules, nevertheless the small molecules are released.

Figure 3. HPLC profiles of solution phase conjugation of RNA **9** with Dox-SS-Py derivative **15**. RP-HPLC profiles of pure RNA **9** (panel a) and crude double DOX RNA conjugate **24** (panel b). Panel c: IEX-chromatogram and MALDI-TOF mass spectrum of pure RNA conjugate **24**.

Here, the disulfide-linked Dox-siRNA conjugates were designed to simultaneously deliver both molecules to cancer cells and lipophilic Dox can be expected to increase siRNA uptake. The double Dox-conjugation with RNA **9** was performed in the same conditions as for the preparation of single Dox-conjugate **22** (Table 2). After 5 min, HPLC analysis of the crude product showed one major peak (71%) corresponding to the desired conjugate **24** (Figure 3b). After purification, double Dox-RNA **24** was obtained with high purity in acceptable yield (38%) (Figure 3c). The MALDI-TOF analysis exhibited a peak ($m/z = 8152.9$) assigned to **24** and a second peak ($m/z = 7754.3$) resulting from the mass fragmentation of Dox during analysis. As previously reported, this peak corresponds to the loss of the amino sugar moiety (loss of 398).^[23] The successful synthesis of this double-Dox RNA conjugate **24** lets expect that the multiple-sites conjugation should be efficient using method B.

Glutathione treatment. The reduction-responsive feature of the RNA-small molecule conjugates **17-23** was tested in the presence of glutathione (GSH) which is the most abundant thiol in the cytosol (up to 10 mM) whereas it is found at low concentration in extracellular medium.

GSH sensitivity of all the conjugates **17-23** were evaluated with 5.6 mM GSH in HEPES buffer (pH 8) by MALDI-TOF mass spectrometry. After 5 min incubation, as shown in Figure 4, the

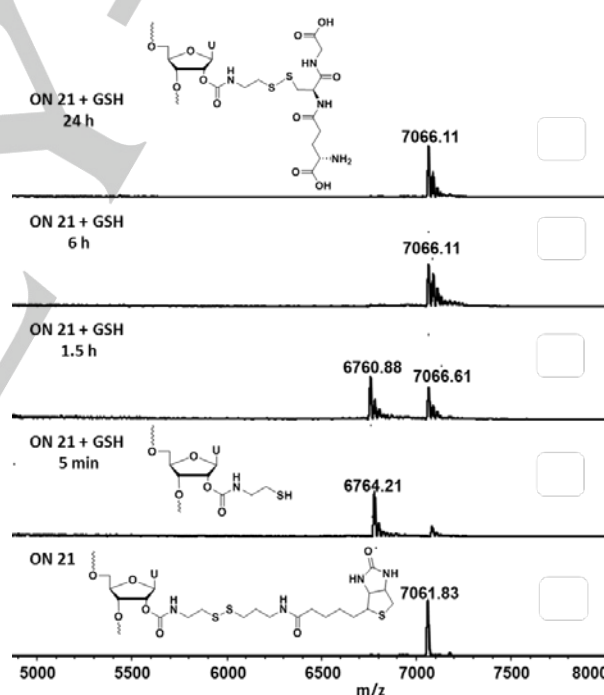


Figure 4. MALDI-TOF MS spectra of RNA-biotin conjugate **21** incubated in the presence of 5.6 mM GSH in HEPES buffer (pH 8) at 37°C, over 24h.

Conclusions

FULL PAPER

In this work, a large variety of small molecules were successfully conjugated to RNA using a disulfide linker attached to a 2'-OH uridine via a carbamate function. The 2'-functionalization of RNA was achieved through the 2'-O-*M*[(acetylthio)-ethylcarbamoyl] uridine phosphoramidite, synthesized in 6 steps with 51% overall yield. Then, the thiol precursor in RNA was activated on solid support with a pyridyldisulfanyl group allowing subsequent thiol-disulfide exchange reaction in solution with diverse small molecules bearing a thiol function. Moreover, the successful preparation of a RNA bearing two Dox molecules attests the great potential of this method for conjugation at multiple and well-defined sites. The rapid cleavage of the disulfide bridge upon 5.6 mM GSH shows that such RNA conjugates will be responsive to the reducing environment of the intracellular medium. The small-molecules will be released with a wide field of applications such as cell-imaging, *in vitro* detection or drug delivery. Particularly, in cancer therapy a co-delivery system based on multiple doxorubicin attached to siRNA via a disulfide bond might be envisaged to boost the anticancer activity and overcome multidrug resistance.

Experimental Section

General Methods. Pyridine, triethylamine and DIEA were distilled over calcium hydride and methanol was distilled over sodium. All other solvents and reagents were purchased from commercial suppliers and were used without further purification. Thin-layer chromatography (TLC) analyses were carried out on silica plate 60 F₂₅₄. Purifications by column chromatography were performed using 0.040-0.063 mm silica from Merck. NMR experiments were recorded on a Bruker 400 spectrometer at 20°C. HRMS analyses were obtained with electrospray ionization (ESI) in positive mode on a Q-TOF Micromass spectrometer. RNAs were synthesized using an automated DNA synthesizer (Applied Biosystems 394). Crude RNAs and conjugates **8-21** were analyzed and purified by IEX-HPLC Dionex DNAPac® PA100, 4 x 250 mm for analysis or 9 x 250 for semi-preparative purpose. The following HPLC solvent system was used: 5% CH₃CN in 25 mM Tris-HCl pH 8 (buffer A), 5% CH₃CN containing 400 mM NaClO₄ in 25 mM Tris-HCl pH 8 (buffer B). Flow rates were 1.0 mL/min for analysis or 4 mL/min for semi-preparative purpose; UV detection was performed at 260 nm. Crude RNA conjugates **22-24** were analyzed and purified by RP-HPLC (Thermo Scientific Accucore aQ C₁₈ 2.6 μm, 2.1 x 50 mm). The following HPLC solvent systems were used: 1% CH₃CN in 12.5 mM TEEAc (buffer A), 80% CH₃CN in 12.5 mM TEEAc (buffer B). Flow rates were 1.9 mL/min. UV detection was performed at 260 nm. MALDI-TOF mass spectra were recorded using a Shimadzu AXIMA Assurance spectrometer equipped with an N₂ laser (337 nm) (Shimadzu, Japan) using 2,1,1-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 the 1:5 (v/v) ratio, crystallized on a 384-well stainless steel plate and analyzed. UV quantitation of RNAs was performed using a Varian Cary 300 Bio UV/Visible spectrometer by measuring absorbance at 260 nm and at 481 nm for RNA conjugates **22** and **24**. Lyophilized RNAs were stored at -20°C for several months without any degradation.

2'-O-(4-nitrophenoxycarbonyl)-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl) uridine (2). A solution of 3',5'-O-

(tetraisopropylidisiloxane-1, 3-diyl) uridine **1** (5.00 g, 10.28 mmol, 1.00 equiv) in dry toluene (100 mL) was treated under argon with dry pyridine (0.82 mL, 10.28 mmol, 1.00 equiv) and 4-nitrophenyl chloroformate (2.38 g, 11.82 mmol, 1.15 equiv). The mixture was stirred for 3 h at room temperature. Then, dry pyridine (0.15 mL, 2.06 mmol, 0.20 equiv) and 4-nitrophenyl chloroformate (0.475 g, 2.06 mmol, 0.20 equiv) were added. After stirring at room temperature for 2 h, the mixture was diluted in ethyl acetate and washed with water. The aqueous layer was then extracted with ethyl acetate. The organic layer was washed with brine and dried over Na₂SO₄. The solvent was evaporated under reduced pressure. The crude material was purified by silica gel column chromatography with cyclohexane/ethyl acetate (70/30). The desired compound **2** was obtained as white foam (5.4 g, 8.2 mmol, 81 %). ¹H NMR (CDCl₃, 400 MHz) δ ppm: 9.14 (s, 1 H, NH), 8.28 (d, *J* = 8.0 Hz, 2 H, Ph), 7.75 (d, *J* = 8.0 Hz, 1 H, H₆), 7.38 (d, *J* = 8.0 Hz, 2 H, Ph), 5.94 (s, 1 H, H₁), 5.74 (d, *J* = 8.4 Hz, 1 H, H₅), 5.32 (d, *J* = 9.2 Hz, 1 H, H₂), 4.47 (dd, *J* = 9.2 Hz, *J* = 3.6 Hz, 1 H, H₃), 4.26 (d, *J* = 10.0 Hz, 1 H, H₅'), 4.11 (d, *J* = 3.6 Hz, 1 H, H₄'), 4.02 (d, *J* = 10.2 Hz, 1 H, H₅'), 1.10-0.98 (m, 28 H, iPr) ppm. ¹³C NMR (CDCl₃, 100 MHz) δ ppm: 163.0, 155.4, 151.4, 149.8, 145.5, 139.0, 125.4, 121.7, 102.4, 88.0, 82.0, 80.2, 67.8, 59.1, 17.4 - 16.7 (8 C), 13.8 - 12.5 (4 C) ppm. HRMS (ESI⁺): *m/z* calcd. for C₂₈H₄₂N₃O₁₁Si₂: 652.2358 [M+H]⁺, found 652.2365.

2'-O-[N-(2-bromoethylcarbamoyl)]-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl) uridine (3). A solution of compound **2** (2.38 g, 3.65 mmol, 1.00 equiv) in dry pyridine (36.5 mL) was treated under argon with 2-bromoethylamine hydrobromide (0.897 g, 4.38 mmol, 1.2 equiv) and dry triethylamine (0.5 mL, 7.3 mmol, 2 equiv). After stirring at room temperature for 4 h, the mixture was diluted in ethyl acetate and washed with water. The aqueous layer was then extracted with ethyl acetate. The combined organic extracts were washed with brine and dried over Na₂SO₄. The solvent was removed under reduced pressure. The crude material was purified by silica gel column chromatography with a step gradient of dichloromethane and ethyl acetate (10-30 %). The desired compound **3** was obtained as white foam (2.27 g, 3.57 mmol, 98 %). ¹H NMR (CDCl₃, 400 MHz) δ ppm: 9.23 (s, 1 H, NH), 7.65 (d, *J* = 4.1 Hz, 1 H, H₆), 5.83 (s, 1 H, H₁), 5.69 (dd, *J* = 4.1 Hz, *J* = 1.1 Hz, 1 H, H₅), 5.67 (t, *J* = 5.5 Hz, 1 H, NH), 5.27 (d, *J* = 5.1 Hz, 1 H, H₂), 4.35 (dd, *J* = 5.1 Hz, *J* = 3.1 Hz, 1 H, H₃), 4.19 (d, *J* = 7.1 Hz, 1 H, H₅'), 3.99-3.96 (m, 2 H, H₅, H₄'), 3.66-3.51 (m, 2 H, CH₂), 3.45 (t, *J* = 5.3 Hz, 2 H, CH₂), 1.08-0.99 (m, 28 H, iPr). ¹³C NMR (CDCl₃, 100 MHz) δ ppm: 163.2, 154.4, 149.7, 139.3, 102.2, 88.5, 82.1, 76.1, 67.9, 59.7, 42.8, 32.2, 16.7-17.4 (8 C), 12.5-13.4 (4 C). HRMS (ESI⁺): *m/z* calcd for C₂₄H₄₂BrN₃O₈Si₂ [M+H]⁺: 636.1772, found 636.1779.

2'-O-[N-(2-acetylthio)-ethylcarbamoyl]-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl) uridine (4). Under argon, compound **3** (4.2 g, 6.6 mmol, 1.00 equiv) was dissolved in dry acetonitrile (132 mL) at 40°C. The solution was treated with potassium thioacetate (1.12 g, 9.9 mmol, 1.5 equiv) and 18-crown-6 (0.871 g, 3.3 mmol, 0.5 equiv). After stirring at room temperature overnight, the mixture was diluted in ethyl acetate and washed with water. The aqueous layer was then extracted with ethyl acetate. The combined organic extracts were washed with brine and dried over Na₂SO₄. The solvent was evaporated under reduced pressure. The crude material was purified by silica gel column chromatography with a step gradient of dichloromethane and ethyl acetate (0-20 %). The desired compound **4** was obtained as white foam (3.36 g, 5.32 mmol, 80 %). ¹H NMR (CDCl₃, 400 MHz) δ ppm: 8.68 (s, 1 H, NH), 7.63 (d, *J* = 8.4 Hz, 1 H, H₆), 5.80 (s, 1 H, H₁), 5.69 (d, *J* = 8.0 Hz, 1 H, H₅), 5.25 (d, *J* = 4.4 Hz, 1 H, H₂), 5.14 (s, 1 H, NH), 4.36 (dd, *J* = 7.8 Hz, *J* = 5.4 Hz, 1 H, H₃), 4.18 (d, *J* = 11.1 Hz, 1 H, H₅'), 4.00-3.95 (m, 2 H, H₅', H₄'), 3.37-3.35 (m, 2 H CH₂), 3.00 (t, *J* = 6.1 Hz, 2 H, CH₂), 2.34 (s, 3 H, CH₃), 1.08-0.95 (m, 28 H, iPr). ¹³C NMR (CDCl₃, 100 MHz) δ ppm: 195.8, 162.8, 154.5, 149.5, 139.5, 102.2, 88.7, 82.1, 75.3, 68.0, 53.4, 40.9, 30.6-

30.9, 29.6, 16.0 – 17.4 (8C), 12.6 – 13.4 (4C). HRMS (ESI⁺): *m/z* calcd for C₂₆H₄₆N₄O₈SSi₂ [M+H]⁺: 632.2493, found 632.2493.

2'-O-[N-(2-acetylthio)-ethylcarbamoyl] uridine (5). Under argon, compound **4** (3.36 g, 5.3 mmol, 1.00 equiv) was dissolved in dry THF (74.5 mL). The solution was treated dropwise with triethylamine trihydrofluoride (1.7 mL, 10.6 mmol, 2 equiv). After stirring 4 h at room temperature, the deprotection was complete and the reaction mixture was treated with triethylammoniumacetate buffer (2 M, pH 7), then co-evaporated with water and acetonitrile. The crude material was purified by silica gel column chromatography with dichloromethane and methanol (95/5). The desired compound **5** was obtained as white foam (1.9 g, 4.88 mmol, 92 %). ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 11.32 (s, 1 H, NH), 7.89 (d, *J* = 8.1 Hz, 1 H, H₆), 7.50 (t, *J* = 6.0 Hz, 1 H, NH), 5.97 (d, *J* = 6.3 Hz, 1 H, H₁), 5.66 (d, *J* = 8.2 Hz, 1 H, H₅), 5.47 (t, *J* = 5.2 Hz, 1 H, OH), 5.18 (t, *J* = 4.6 Hz, 1 H, H₂), 5.02 (t, *J* = 5.7 Hz, *J* = 5.1 Hz, 1 H, H₃), 4.19 (dd, *J* = 8.5 Hz, *J* = 4.9 Hz, 1 H, H₄), 3.88 (d, *J* = 3.0 Hz, 1 H, OH), 3.65–3.54 (m, 2 H, H₅ + H_{5'}), 3.25–3.09 (m, 2 H, CH₂), 2.94–2.84 (m, 2 H, CH₂), 2.31 (s, 3 H, CH₃). ¹³C NMR (DMSO-d₆, 100 MHz) δ ppm: 195.6, 163.5, 155.6, 151.0, 141.1, 102.6, 86.2, 85.9, 75.2, 69.4, 61.4, 40.9, 31.0, 28.8. HRMS (ESI⁺): *m/z* calcd for C₁₄H₁₉N₃O₈S [M+H]⁺: 390.0971, found 390.0972.

2'-O-[N-(2-acetylthio)-ethylcarbamoyl]-5'-O-(4,4'-dimethoxytrityl) uridine (6). Compound **5** (0.532 g, 1.37 mmol, 1 equiv) was dried for 24 h under reduced pressure, then was dissolved under argon in dry pyridine (2.6 mL) at room temperature. After complete dissolution, dry DCM (13.7 mL) was added. The solution was treated with *N*,*N*-diisopropylethylamine (DIEA) (263 μL, 1.51 mmol, 1.1 equiv) and dimethoxytrityl chloride (DMTrCl) (559 mg, 1.65 mmol, 1.2 equiv) added in small portions over 1 h. After 2 h and 6 h, DIEA and DMTrCl (0.5 and 0.2 equiv respectively) were added to the reaction. Completion was reached after 7 h, the mixture was diluted in DCM and poured into saturated NaHCO₃ solution. The aqueous layer was then extracted with DCM. The combined organic extracts were washed with brine and dried over Na₂SO₄. The solvent was removed under reduced pressure. The crude material was purified by silica gel column chromatography with DCM and pyridine (99/1), then DCM with methanol (1–5 %). The desired compound **6** was obtained as pale yellow foam (980 mg, 1.34 mmol, 98 %). ¹H NMR (CDCl₃, 400 MHz): δ ppm: 9.36 (s, 1 H, NH), 7.75 (d, *J* = 8.2 Hz, 1 H, H₆), 7.40–7.16 (m, 9 H, H_{Ar}), 6.84 (d, *J* = 8.9 Hz, 4 H, H_{Ar}), 6.17 (d, *J* = 5.1 Hz, 1 H, H₁), 5.80 (t, *J* = 6.1 Hz, 1 H, NH), 5.34 (dd, *J* = 8 Hz, *J* = 1.2 Hz, 1 H, H₅), 5.24 (t, *J* = 5.1 Hz, 1 H, H₂), 4.67 (dd, *J* = 7.6 Hz, *J* = 4.0 Hz, 1 H, H₃), 4.17 (d, *J* = 3.1 Hz, 1 H, H₄), 3.79 (s, 6 H, OCH₃), 3.57 (m, 1 H, H₅), 3.57–3.48 (m, 2 H, H₅' + HCH), 3.25–2.89 (m, 1 H + 2 H, HCH + CH₂), 2.15 (s, 3 H, CH₃). ¹³C NMR (CDCl₃, 100 MHz) δ ppm: 196.9, 163.3, 158.7, 155.4, 150.7, 144.2, 140.2, 137.9, 135.9, 135.0, 130.2, 130.2, 130.0, 129.1, 129.1, 128.2, 128.2, 128.1, 128.1, 127.2, 125.3, 113.4, 113.3, 102.8, 87.2, 86.0, 84.1, 70.3, 62.8, 55.3, 55.3, 41.2, 28.8, 21.5. HRMS (ESI⁺): *m/z* calcd for C₃₅H₃₈N₃O₁₀S [M+H]⁺: 692.2200, found 692.2283.

2'-O-[N-(2-acetylthio)-ethylcarbamoyl]-3'-O-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite)-5'-O-(4,4'-dimethoxytrityl) uridine (7) Under argon, compound **6** (0.850 g, 1.23 mmol, 1 equiv) was dissolved in anhydrous CH₂Cl₂ (10 mL) previously passed through an alumina column. A mixture of DIEA (386 μL, 2.21 mmol, 1.8 equiv) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (437 μL, 1.95 mmol, 1.5 equiv) in CH₂Cl₂ (2.3 mL) was added dropwise. The mixture was stirred at room temperature for 1.5 h. DIEA (129 μL, 0.74 mmol, 0.6 equiv) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (137 μL, 0.65 mmol, 0.5 equiv) in CH₂Cl₂ (2.3 mL) was added dropwise, and the reaction was stirred at room temperature for 2 h. Then, the mixture was diluted with ethyl acetate and washed with a saturated aqueous NaHCO₃ solution. The aqueous phase was extracted with ethyl acetate. The combined organic layers were dried over Na₂SO₄ and evaporated under reduced pressure.

The crude residue was purified by silica gel column chromatography with an isocratic elution of CH₂Cl₂/EtOAc (4/6) containing 1 % pyridine. The desired compound **7** was obtained as a white foam. (975 mg, 1.09 mmol, 89 %). ¹H NMR (CD₃CN, 400 MHz): δ ppm: 9.19 (s, 1 H, NH), 7.75 (d, *J* = 7.6 Hz, 1 H, H₆), 7.50–7.47 (m, 2 H, H_{Ar}), 7.38–7.33 (m, 5 H, H_{Ar}), 7.23–7.18 (m, 2 H, H_{Ar}), 6.90 (dd, *J* = 8.6 Hz, *J* = 3.8 Hz, 4 H, H_{Ar}), 6.05–5.95 (m, 2 H, H₁' + NH), 5.43–5.36 (m, 2 H, H₅ + H₂), 4.68–4.59 (m, 1 H, H₃), 4.23 (dd, *J* = 24.3 Hz, *J* = 2.6 Hz, 1 H, H₄), 3.93–3.83 (m, 1 H, H₅), 3.80 (s, 3 H, OCH₃), 3.79 (s, 3 H, OCH₃), 3.76–3.66 (m, 1 H, H₅'), 3.64–3.56 (m, 2 H, CH₂), 3.41 (dd, *J* = 15.0 Hz, *J* = 2.8 Hz, 2 H, CH₂), 3.30 (h, *J* = 6.9 Hz, 1 H, CH-(CH₃)₂), 3.22 (h, *J* = 6.8 Hz, 1 H, CH-(CH₃)₂), 3.08–2.92 (m, 2 H, CH₂), 2.72 (t, *J* = 6.0 Hz, 1 H, HCH), 2.53 (t, *J* = 6.0 Hz, 1 H, HCH), 2.36 (s, 3 H, CH₃), 1.19–1.06 (m, 12 H, 4 CH₃). ¹³C NMR (CD₃CN, 100 MHz) δ ppm: 196.0, 163.4, 159.4, 155.6, 151.0, 150.3, 145.3, 145.2, 140.8, 140.7, 138.5, 136.5, 136.0, 135.9, 135.7, 130.7, 129.5, 128.8, 128.6, 127.6, 125.8, 124.3, 113.8, 102.7, 87.1, 86.9, 84.0, 75.3, 71.5, 71.0, 63.4, 59.6, 58.8, 55.5, 43.5, 40.9, 30.4, 29.2, 24.7, 24.6, 24.5, 24.4, 21.0, 20.6. ³¹P-NMR (CD₃CN, 162 MHz) δ ppm: 149.66, 149.58. HRMS (ESI⁺): *m/z* calcd for C₄₄H₅₄N₅O₁₁PS [M+H]⁺: 892.3278, found 892.3345.

Solid-phase synthesis of RNA 8 and 9. RNA oligonucleotides were synthesized using an ABI model 394 DNA/RNA synthesizer on 1 μmol scale using commercial 2'-O-PivOM ribonucleosides phosphoramidites (Chemgenes) or 2'-O-[N-(acetylthioethyl)carbamoyl] uridine phosphoramidite **7** and a long chain alkylamine (LCAA) controlled-pore glass (CPG) as solid support through a succinyl linker. 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 the activator. The oxidizing solution was 0.1 M iodine in THF/pyridine/H₂O (78:20:2, v/v/v) (Link Technologies). The capping step was performed with a mixture of 5% phenoxyacetic anhydride (Pac₂O) in THF and 10% *N*-methylimidazole in THF (Link Technologies). Detritylation was performed with 3% TCA in CH₂Cl₂. After RNA assembly completion, the column was removed from the synthesizer and dried under a stream of argon.

The solid support was treated with 2 mL of a 0.4 M solution of 2,2'-dithiodipyridine (1000 equiv. per modification) and a 2.5 M butylamine (0.5 mL) solution in anhydrous THF (1.5 mL). The solution was applied to the synthesis column using two glass syringes filled with 4 Å molecular sieves (5 beads each), and was pushed back and forth through the synthesis column for 15 min. Then the solution was removed and the solid-support was washed with anhydrous THF followed by a 1 min flush with argon. Finally, the solid support was treated with a 30 % aqueous ammonia solution for 3 h at 25°C. The solution was evaporated in the presence of isopropylamine (13 % of total volume) under reduced pressure. The residue was dissolved in 1.5 mL water, transferred to a 2 mL Eppendorf-vial and lyophilized.

Analysis, purification and desalting of RNA 8 and 9. The crude RNAs were then purified by semi-preparative IEX-HPLC with a 20 min linear gradient of 0% to 60% of eluent B in eluent A. The pure fractions of each RNA were pooled in a 100 mL round-bottomed flask and were concentrated to dryness under reduced pressure. The residues were dissolved in 100 mL TEAAc buffer, pH 7 and they were desalted using a C₁₈ cartridge (Sep-Pak®, Waters) equilibrated with a 100 mM TEAAc buffer solution. The desired compound was eluted with a 12.5 mM TEAAc/CH₃CN (50/50) solution in a 50 mL round-bottomed flask and was lyophilized. The residue was dissolved in 1.5 mL water (divided in 3 portions 0.8 mL, 0.4 mL and 0.3 mL for flask rinsing), transferred to a 2 mL Eppendorf-vial and lyophilized. Purified RNAs **8** and **9** were characterized by MALDI-TOF MS and quantified by UV measurement

Procedures for conjugation of RNAs in solution.

Preparation of RNA-sugar conjugates 17, 18 and RNA-GSH 19. A 10 mM solution of 1-thio- β -D-glucose **10**, 1-thio- β -D-galactose **11**, or glutathione **12** was prepared in HEPES buffer (34 mM NaCl, 1 mM MgCl₂, 0.05 mM EDTA, 7.5 mM HEPES, pH 8). The buffered solution (10 μ L by nmol of RNA) was added to the lyophilized RNA **8** in a 0.5 mL Eppendorf. The mixture was stirred at 37°C for 24 h using a thermoshaker (Thermal Shake lite®). The reaction was stopped by freezing in liquid nitrogen. The RNA conjugates **17**, **18** and **19** were purified by IEX-HPLC, desalted and characterized by MALDI-TOF MS using the procedures described above.

Preparation of RNA-4-methylcoumarin conjugate 20. A 10 mM solution of 7-Mercapto-4-methylcoumarin **13** was prepared in 1:1 mixture of HEPES buffer (34 mM NaCl, 1 mM MgCl₂, 0.05 mM EDTA, 7.5 mM HEPES, pH 8) and DMF. The buffered solution (10 μ L by nmol of RNA) was added to the freeze-dried RNA **8** in a 0.5 mL Eppendorf. Then, the mixture was stirred at 37°C for 24 h using a thermoshaker. The reaction was stopped by freezing in liquid nitrogen. The RNA-4-methylcoumarin conjugate **20** was purified by IEX-HPLC, desalted and characterized by MALDI-TOF spectrometry using the procedures described above.

Preparation of RNA-biotin conjugate 21. A 10 mM solution of (+) - Biotin *N*-2-(pyridin-2-ylsulfanyl)ethyl amide **14** was prepared in 1:1 mixture of HEPES buffer (34 mM NaCl, 1 mM MgCl₂, 0.05 mM EDTA, 7.5 mM HEPES, pH 8) and DMF. 100 μ L of this solution (1000 nmol, 100 equiv.) were diluted with 84 μ L of HEPES buffer pH 8 and 16 μ L of a buffered solution of TCEP (50 nmol / μ L, 80 equiv.). The mixture was stirred for 2 h at 37°C using a thermoshaker. Then the solution was added to the freeze-dried RNA **8** in a 0.5 mL Eppendorf and stirred at 37°C for 24 h using a thermoshaker. The reaction was stopped by freezing in liquid nitrogen and the RNA-biotin conjugate **21** was purified by IEX-HPLC, desalted and characterized by MALDI-TOF MS using the procedures described above.

Preparation of RNA-Dox conjugates 22 and 24. A 10 mM solution of Doxorubicin 2-(pyridin-2-ylsulfanyl)ethylcarbamate **15** was prepared in DMF. 100 μ L of this solution (1000 nmol, 100 equiv) were diluted with 84 μ L of HEPES buffer (34 mM NaCl, 1 mM MgCl₂, 0.05 mM EDTA, 7.5 mM HEPES, pH 8) and 16 μ L of a buffered solution of TCEP (50 nmol / μ L, 80 equiv). The mixture was stirred for 2 h at 37°C using a thermoshaker. Then the solution was added to the freeze-dried RNA **8** or **9** in a 0.5 mL Eppendorf and stirred at 37°C for 5 min using a thermoshaker. The reaction was stopped by addition of a large excess of HEPES buffer pH 5. The RNA-Dox conjugates **22** and **24** were immediately purified by RP-HPLC, characterized by IEX-HPLC and MALDI-TOF MS using the procedures described above.

Preparation of RNA-deoxycholic acid conjugate 23. A 10 mM solution of *N*-(2-(pyridin-2-ylsulfaneyl)ethyl)-3 α .12 α -dihydroxy-5 β -cholan-24-amide **16** was prepared in 2:3 mixture of HEPES buffer (34 mM NaCl, 1 mM MgCl₂, 0.05 mM EDTA, 7.5 mM HEPES, pH 8) and DMF. 100 μ L of the solution (1000 nmol, 100 equiv) were diluted with 86 μ L of buffer and 16 μ L of a buffered solution of TCEP (50 nmol / μ L, 80 equiv). The mixture was stirred for 2 h at 37°C using a thermoshaker. Then, the solution was added to the freeze-dried RNA **8** in a 0.5 mL Eppendorf and stirred at 37°C for 24 h using a thermoshaker. The reaction was stopped by freezing in liquid nitrogen and the RNA-deoxycholic acid conjugate **23** was purified by RP-HPLC, evaporated and characterized by MALDI-TOF MS using the procedures described above.

Recovery yields for RNA-Dox conjugates **22** and **24** were calculated from UV absorption at 481 nm of the RNA conjugates. Other recovery yields

were calculated from UV absorption at 260 nm of RNA-small molecule conjugates.

Reductive conversion of RNA under glutathione treatment. 1 nmol of RNA-small molecule conjugates (1 nmol) were freeze-dried in 500 μ L Eppendorf tubes then were dissolved in 100 μ L of a glutathione-HEPES buffer (34 mM NaCl, 1 mM MgCl₂, 0.05 mM EDTA, 7.5 mM HEPES, 10 mM glutathione, pH 8) and incubated at 37°C. For RNA-conjugates **17**, **19**, **20**, **21** and **23**, aliquots were collected after 5 min, 1.5 h, 6 h and 24 h. For RNA conjugate **18**, aliquots were collected after 1.5 h, 6 h and 24 h. For RNA conjugate **22**, aliquots were collected after 30 min, 4 h and 24 h. All aliquots were immediately frozen in liquid nitrogen and kept at -80°C until analysis. Samples were diluted with water and analyzed by MALDI-TOF MS.

Acknowledgments

Florian Gauthier thanks the University of Montpellier for financial support.

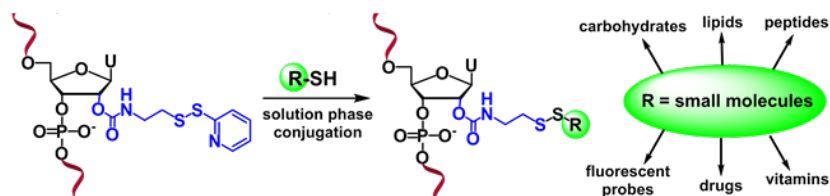
Keywords: conjugation • RNA • disulfide bridge • carbamate • 2'-OH modification

- [1] X. Ming, B. Laing, *Adv. Drug Deliv. Rev.* **2015**, *87*, 81-89.
 [2] a) A. Aviñó, S. M. Ocampo, R. Lucas, J. J. Reina, J. C. Morales, J. C. Perales, R. Eritja, *Mol. Divers.* **2011**, *15*, 751-757; b) B. Ugarte-Urbe, S. Pérez-Rentero, R. Lucas, A. Aviñó, J. J. Reina, I. Alkorta, R. Eritja, J. C. Morales, *Bioconjugate Chem.* **2010**, *21*, 1280-1287; c) T. S. Zatsepin, T. S. Oretskaya, *Chem. Biodivers.* **2004**, *1*, 1401-1417; d) Y. Ikeda, D. Kubota, Y. Nagasaki, *Bioconjugate Chem.* **2010**, *21*, 1685-1690.
 [3] a) S. Matsuda, K. Keiser, J. K. Nair, K. Charisse, R. M. Manoharan, P. Kretschmer, C. G. Peng, A. V. Kel'in, P. Kandasamy, J. L. S. Willoughby, A. Liebow, W. Querbes, K. Yucius, T. Nguyen, S. Milstein, M. A. Maier, K. G. Rajeev, M. Manoharan, *ACS Chem. Biol.* **2015**, *10*, 1181-1187; b) A. D. Springer, S. F. Dowdy, *Nucleic Acid Ther.* **2018**, *28*, 109-118.
 [4] L. He, Q. Xu, Y. Liu, H. Wei, Y. Tang, W. Lin, *ACS Appl. Mater. Interfaces* **2015**, *7*, 12809-12813.
 [5] a) G. Russell-Jones, K. McTavish, J. McEwan, J. Rice, D. Nowotnik, *J. Inorg. Biochem.* **2004**, *98*, 1625-1633; b) W. X. Ren, J. Han, S. Uhm, Y. J. Jang, C. Kang, J.-H. Kim, J. S. Kim, *Chem. Commun.* **2015**, *51*, 10403-10418.
 [6] a) J. R. Dunetz, J. Magano, G. A. Weisenburger, *Org. Process Res. Dev.* **2016**, *20*, 140-177; b) A. V. Kachalova, D. A. Stetsenko, E. A. Romanova, V. N. Tashlitsky, M. J. Gait, T. S. Oretskaya, *Helv. Chim. Acta* **2002**, *85*, 2409-2416.
 [7] a) Y. Singh, E. Defrancq, P. Dumy, *J. Org. Chem.* **2004**, *69*, 8544-8546; b) D. Forget, D. Boturyn, E. Defrancq, J. Lhomme, P. Dumy, *Chem. Eur. J.* **2001**, *7*, 3976-3984.
 [8] a) T. Yamada, C. G. Peng, S. Matsuda, H. Addepalli, K. N. Jayaprakash, M. R. Alam, K. Mills, M. A. Maier, K. Charisse, M. Sekine, M. Manoharan, K. G. Rajeev, *J. Org. Chem.* **2011**, *76*, 1198-1211; b) W. Wang, K. Chen, D. Qu, W. Chi, W. Xiong, Y. Huang, J. Wen, S. Feng, B. Zhang, *Tetrahedron Lett.* **2012**, *53*, 6747-6750; c) M. Gerowska, L. Hall, J. Richardson, M. Shelbourne, T. Brown, *Tetrahedron* **2012**, *68*, 857-864.
 [9] M. H. Lee, Z. Yang, C. W. Lim, Y. H. Lee, S. Dongbang, C. Kang, J. S. Kim, *Chem. Rev.* **2013**, *113*, 5071-5109.
 [10] a) H. Lönnberg, *Bioconjugate Chem.* **2009**, *20*, 1065-1094; b) F. Maurel, F. Debart, F. Cavellier, A. R. Thierry, B. Lebleu, J.-J. Vasseur, E. Vivès, *Bioorg Med Chem Lett* **2005**, *15*, 5084-5087; c) W. H. A. Kuijpers, C. A. A. van Boeckel, *Tetrahedron* **1993**, *49*, 10931-10944; d) Y.-H. Rogers, P. Jiang-Baucom, Z.-J. Huang, V. Bogdanov, S. Anderson, M. T. Boyce-Jacino, *Anal Biochem* **1999**, *266*, 23-30; e) D. R. Corey, *Methods Mol. Biol.* **2004**, *283*, 197-206.
 [11] a) C. He, G. L. Verdine, *Chem Biol* **2002**, *9*, 1297-1303; b) H. Huang, S. C. Harrison, G. L. Verdine, *Chem Biol* **2000**, *7*, 355-364; c) S. R. Paalman, D. M. Noll, N. D. Clarke, *Nucleic Acids Res.* **1997**, *25*, 1795-1801.

- [12] S. Pérez-Rentero, S. Grijalvo, R. Ferreira, R. Erić, *Molecules* **2012**, *17*, 10026.
- [13] M. Dirin, E. Urban, C. R. Noe, J. Winkler, *Eur. J. Med. Chem.* **2016**, *121*, 132-142.
- [14] V. Metelev, A. Romanenkov, E. Kubareva, E. Zubin, N. Polouchine, T. Zatsepin, N. Molochkov, T. Oretskaya, *IUBMB life* **2006**, *58*, 654-658.
- [15] a) A. Biscans, S. Rouanet, J.-J. Vasseur, C. Dupouy, F. Debart, *Org. Biomol. Chem.* **2016**, *14*, 7010-7017; b) F. Gauthier, S. Claveau, J.-R. Bertrand, J.-J. Vasseur, C. Dupouy, F. Debart, *Bioorg. Med. Chem.* **2018**, *26*, 4635-4643.
- [16] F. Gauthier, F. Beltran, A. Biscans, F. Debart, C. Dupouy, J.-J. Vasseur, *Org. Biomol. Chem.* **2018**, *16*, 3181-3188.
- [17] A. K. Ghosh, M. Brindisi, *J. Med. Chem.* **2015**, *58*, 2895-2940.
- [18] a) A. Misra, S. Mishra, K. Misra, *Bioconjugate Chem.* **2004**, *15*, 638-646; b) K. Seio, R. Tawarada, T. Sasami, M. Serizawa, M. Ise, A. Ohkubo, M. Sekine, *Bioorg. Med. Chem.* **2009**, *17*, 7275-7280; c) K. Seio, M. Tokugawa, T. Kanamori, H. Tsunoda, A. Ohkubo, A. Sekine, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 2470-2473; d) M. Prhac, E. A. Lesnik, V. Mohan, M. Manoharan, *Tetrahedron Lett.* **2001**, *42*, 8777-8780; e) N. N. Dioubankova, A. D. Malakhov, D. A. Stetsenko, M. J. Gait, P. E. Volynsky, R. G. Efremov, V. A. Korshun, *ChemBioChem* **2003**, *4*, 841-847.
- [19] T. Lavergne, J.-R. Bertrand, J.-J. Vasseur, F. Debart, *Chem. Eur. J.* **2008**, *14*, 9135-9138.
- [20] a) G. Sengle, A. Jenne, P. S. Arora, B. Seelig, J. S. Nowick, A. Jäschke, M. Famulok, *Bioorg. Med. Chem.* **2000**, *8*, 1317-1329; b) V. Noponen, H. Belt, M. Lahtinen, A. Valkonen, H. Salo, J. Ulrichová, A. Galandáková, E. Sievänen, *Steroids* **2012**, *77*, 193-203.
- [21] A. Latorre, P. Couleaud, A. Aires, A. L. Cortajarena, Á. Somoza, *Eur. J. Med. Chem.* **2014**, *82*, 355-362.
- [22] a) H. Meng, M. Liong, T. Xia, Z. Li, Z. Ji, J. I. Zink, A. E. Nel, *ACS Nano* **2010**, *4*, 4539-4550; b) A. M. Chen, M. Zhang, D. Wei, D. Stueber, O. Taratula, T. Minko, H. He, *Small* **2009**, *5*, 2673-2677.
- [23] D. Kaushik, G. Bansal, *J. Pharm. Anal.* **2015**, *5*, 285-295.

Entry for the Table of Contents

FULL PAPER



In this paper, we developed a post-synthesis conjugation of small molecules to RNA using a reduction sensitive disulfide linker connected to 2'-OH via a carbamate function.

RNA conjugation

F. Gauthier, A. Malher, J.J Vasseur, C. Dupouy, F. Debart**

Page No. – Page No.

Conjugation of small molecules to RNA using a reducible disulfide linker attached at 2'-OH position via a carbamate function.