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A biotin-conjugated pyridine-based isatoic anhydride, a selective room temperature RNA-acylating agent for the nucleic acid separation†

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Isatoic anhydride derivatives, including a biotin and a disulfide linker were specifically designed for nucleic acid separation. 2′-OH selective RNA acylation, capture of biotinylated RNA adducts by streptavidin-coated magnetic beads and disulfide chemical cleavage led to isolation of highly enriched RNA samples from an initial 9/1 DNA–RNA mixture. Starting from the parent compound *N*-methylisatoic anhydride **A** which was used at 65 °C, we improved the extraction process by designing a new generation of isatoic anhydrides that are able to react under smoother conditions. Among them, a pyridine-based isatoic anhydride derivative **15f** was found to be reactive at room temperature, leading to enhance the efficiency and selectivity of the extraction process by significantly reducing DNA side extraction. The extracted and purified RNAs can then be detected by RT-PCR.

Introduction

Extraction and purification of nucleic acids from a biological sample, called Sample Prep, is a crucial step in molecular diagnostics. This process is essential to remove interfering substances and isolate nucleic acids of satisfactory quality.[1a,b] The majority of commercial methods involve the use of silica matrices, referred as "Boom chemistry".[2] With high chaotropic salt concentration, nucleic acids can bind silica and thus be separated from other cellular components. This technique which led to the automation of the Sample Prep step does not allow selective extraction of each nucleic acid target. Regarding RNA, the contamination by predominant genomic DNA is the cause of many issues for RNA detection and analysis. Purity and integrity of RNA are two critical parameters for many applications based on RNA detection by RT-PCR.[3]

The close chemical and physical properties of the two nucleic acid targets make the specific RNA extraction an intricate process, and several methods have been developed to isolate RNAs from biological samples. The primary procedure used in molecular biology laboratories is based on an extraction process using a guanidinium thiocyanate–phenol–chloroform mixture.[4a,b] The use of organic solvents and the necessity to add a DNase step to remove residual genomic DNA before analysis by RT-PCR[5a,b] makes the method time-consuming and very difficult to automate. New methods to isolate pure RNA from biological samples have thus recently been developed. For example, liquid chromatography, and particularly arginine matrices, have recently been applied to RNA isolation from eukaryotic cells.[6] Besides separation processes based on physical properties of nucleic acids, chemical methods based on selective nucleic acid functionalization could be an alternative. Reversible boronic ester formation between boronic acids and the *cis*-diol group of ribose has been used for ribonucleoside, oligoribonucleotide and RNA purification. Boronic acids, and particularly phenyl boronic acid-modified cellulose[7] and polyacrylamide[8a,b] have been developed for this application. However, this method was not evaluated for the nucleic acid separation from biological samples.[9]

We have recently described a biotin-conjugated *N*-methyl isatoic anhydride **A** (NMIA), designed to purify RNAs by selective 2′-OH acylation of ribose, capture by streptavidin-coated magnetic beads and release through a disulfide bond cleavage (Fig. 1).[10] This reagent allowed the selective extraction of RNA from a DNA–RNA mixture, and the extracted RNA can be successfully amplified by RT-PCR. However, the conditions of acylation used to capture significant amounts of RNAs (65 °C at 1.5 mM of **A**) led to the presence of residual DNA. In order to

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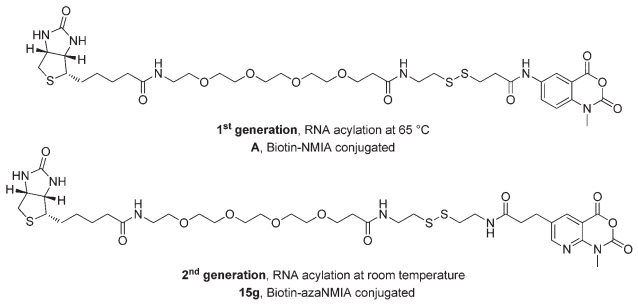


Fig. 1 Biotin-isatoic anhydride conjugated designed for RNA selective extraction.

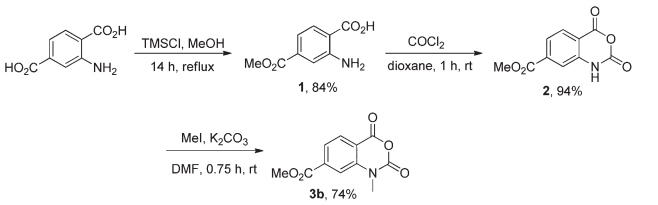
improve the selectivity of the acylation step, and hence decrease the ratio of extracted DNA, we were interested in the development of more reactive isatoic anhydride (IA) derivatives which could be able to react in smoother conditions to avoid side reactions with DNA, while maintaining or improving the efficiency for RNA extraction.

The enhancement of reactivity of IA derivatives toward 2′-OH RNA acylation being the result of the higher electrophilic character of the anhydride carbonyl group,[11] we were able to design more reactive RNA-acylating agents after a primary screening of IA derivatives. Among them, we found that a pyridine-based isatoic anhydride **15f** was able to react with RNA at room temperature, leading to a better selectivity in the extraction protocol compared to the parent compound **A**.

Results and discussion

IA derivatives bearing electron withdrawing groups (**3b–3f**) were synthesized in order to improve RNA selective acylation and were compared to the parent NMIA (**3a**) and to an isatoic anhydride containing electron donating groups (**3i**). Heterocyclic analogues containing a π-deficient pyridine (**3g**) or a π-excessive thiophene ring (**3h**) were synthesized for comparison.

Except for compound **3b** bearing a 7-carboxy group, all the anhydrides **3c–i** were obtained using literature methods (Fig. 2).[10–15] The synthesis of anhydride **3b** was achieved through a three-step sequence starting from 2-aminoterephthalic acid. The selective esterification of carboxylic acid at position 4 using chlorotrimethylsilane in methanol led to the monoester **1** which was subsequently treated with phosgene and *N*-methylated with iodomethane to give **3b** in 58% overall yield (Scheme 1).



Scheme 1 Synthesis of isatoic anhydride **3b**.

IA derivatives **3b–3i** were then tested toward acylation of short synthetic 27-nt RNAs and the results were compared to the parent NMIA **3a**. According to our previously reported methodology, an RNA oligomer was incubated with isatoic anhydride derivatives **3a–3i** in a DMSO–TEAAc mixture (25/75, pH7) at 65 °C for 1 h. After removing the tag excess, RNA oligomers were subjected to an enzymatic hydrolysis and the resulting mixture of nucleosides was analyzed by HPLC. The ratios of tagged nucleosides *vs.* non-tagged were determined and the efficiency of the acylation process was compared for each IA derivative. Results are depicted in Table 1.

As expected, the introduction of electron donating groups (**3i**) or the π-excessive thiophene ring (**3h**) led to a decrease of the acylation ratio, whereas the IA derivatives containing an electron withdrawing group at position 7 (**3b–d**) led to an increase of this ratio compared to **3a**. When electron withdrawing groups were placed at position 6 (**3e–f**), the reactivity was similar to that of compound **3a**. All these results are in accordance with the electronic effects of substituents in aromatic series. The electrophilic character of the reactive carbonyl group of IA is indeed increased when strong electron withdrawing groups are placed at the *para* position. Interestingly the introduction of a pyridine (compound **3g**) led to acylation ratios similar to those obtained for the 7-carboxy derivative **3b**, respectively 9.7 and 9.4%. For these two compounds, we investigated the acylation process at room temperature. Under these conditions 8-aza-NMIA **3g** remained reactive, leading to a slight decrease of the acylation ratio (7%) whereas the reactivity of the parent NMIA **3a** dramatically decreased to only 1.3%

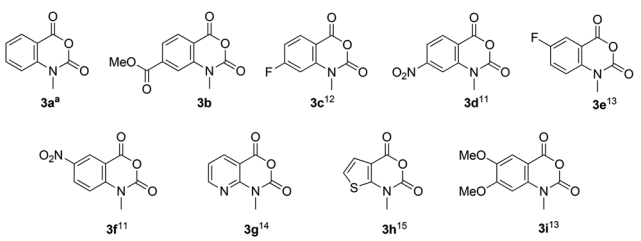


Fig. 2 Selected isatoic anhydride **3a–i**. [a]NMIA (*N*-Methylisatoic-Anhydride) is commercially available.

Table 1 Synthetic 27-nt RNA tagging with **3a–i**

Tag	Tagged nucleosides[a] (%) at 65 °C	Tagged nucleosides[a] (%) at 25 °C
3a	6.1	0.7
3b	9.7	1.3
3c	8.1	nd[b]
3d	7.1	nd[b]
3e	6.1	nd[b]
3f	6.6	nd[b]
3g	9.4	7.0
3h	2.0	nd[b]
3i	2.0	nd[b]

[a] Ratio determined by LC/MS, see experimental data for details. [b] nd: non-determined.

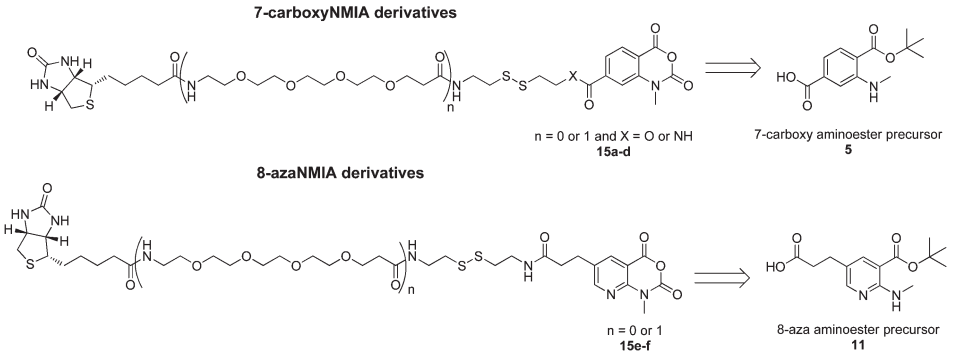


Fig. 3 7-Carboxy and 8-azaisatoic anhydride derivatives **15a–f** functionalized for RNA selective extraction.

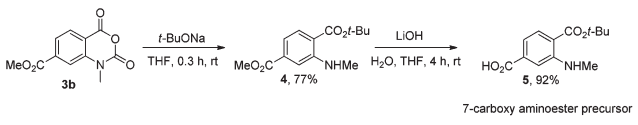
of acylated nucleosides. Despite the higher reactivity at 65 °C of 7-carboxyNMIA **3b**, this compound was not more efficient than NMIA at room temperature with only 1.3% of acylated nucleosides. In these experiments at room temperature, we observed that compound **3b** remained poorly soluble in the reaction mixture, whereas the pyridine derivative **3g** showed good solubility. These results highlighted the great importance of water solubility for an efficient RNA acylation. We then selected the pyridine and the 7-carboxy derivatives and synthesized the biotin-peg$_4$-disulfide-conjugated compounds to further study their use in the RNA extraction and purification. The lower reactivity of the 7-carboxy derivative should be compensated by the introduction of a hydrophilic peg linker on the structure leading to more water-soluble compounds.

The chemical strategy to introduce the biotin-peg$_4$-disulfide moiety was the same as previously described for the parent NMIA **A**. For the 7-carboxy derivative, the biotin conjugate was directly coupled to the carboxylic acid group through either an amide or an ester bond whereas the pyridine ring was previously functionalized with a propanoyl chain. The chemical synthesis required the preparation of the *tert*-butylaminoester precursors **5** and **11** (Fig. 3). Each conjugated anhydride was prepared with and without the peg$_4$ linker in order to compare the influence of hydrosolubility on the RNA acylation reaction. Starting from 7-carboxyNMIA derivative **3b**, *tert*-butylaminoester **4** was obtained by opening the oxazinone ring with sodium *tert*-butoxide (Scheme 2). The latter compound was then subjected to selective hydrolysis by the methyl ester group using lithium hydroxide at room temperature leading to the 7-carboxy aminoester precursor **5** in a good overall yield.

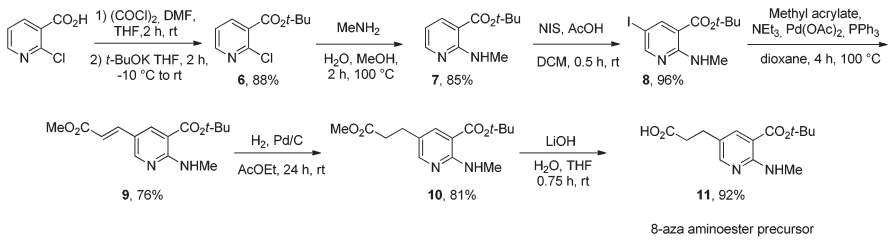
The synthesis of 8-aza aminoester precursor **11** began with the protection of the acid as its *tert*-butyl ester followed by a SNAr with methylamine to give **7** in good yield. The propionyl chain was then introduced following a selective electrophilic iodination at position 5 and a subsequent Heck cross-coupling reaction with methylacrylate, using a combination of Pd(OAc)$_2$ and PPh$_3$. The catalytic hydrogenation of the alkene moiety followed by the selective hydrolysis of the methylester with lithium hydroxide led to the azaisatoic precursor **11** in a 41% overall yield over 6 steps (Scheme 3).

The disulfide linker was introduced through a cystamine for the pyridine and the 7-carboxamidoisatoic derivatives. Cystamine was first protected as its mono-*N*-Boc derivative **I** according to the procedure described by Niu J. *et al.*[16] For the ester of 7-carboxy derivative, we prepared a dissymmetric disulfide linker **II** bearing a hydroxyl group from 2-mercaptoethylamine according to the procedure described by Nemmani K.V.S. *et al.*[17] The two carboxylic acids **5** and **11** were activated using EDC and HOBt in DCM. The addition of the disulfide linkers **I** and **II** led to the formation of the corresponding amides **12b–c** and ester **12a** which were deprotected with TFA in DCM to give the free amines **13a**, **13b** and **13c** (Scheme 4). The amines **13a–c** were then conjugated to a biotin activated as *N*-hydroxysuccinimide ester and a biotin-peg$_4$ activated as a pentafluorophenyl ester. The resulting biotinylated derivatives **14a–f** were obtained in yields ranging between 70 and 90%. The final anhydride derivatives **15a–d** were obtained by treatment with phosgene in DCM from the corresponding 7-carboxy aminoesters **14a–d** in 50–85% yields. For the pyridine anhydrides **15e–f**, DCM was replaced by diethyl ether as the solvent, and the addition of triethylamine was necessary to avoid protonation of the basic 2-aminopyridine ring. With this procedure, the non-pegylated anhydride **15e** was isolated in 50% yield. Starting from the pegylated aminoester **14f**, the formation of non-soluble aggregates in Et$_2$O hampered the cyclization reaction. The anhydride **15g** was obtained in 25% yield after previous absorption of **14f** on C$_{18}$-RP-silica before treatment with phosgene in the presence of TEA (Scheme 5). Starting from 2-aminoterephthalic acid, 7-carboxy anhydrides **15a–d** were successfully obtained in 9 steps with overall yields of 24, 28, 18 and 38%, respectively. In a similar manner, azaisatoic anhydrides **15e–f** were obtained in 10 steps starting from 2-chloronicotinic acid in 11 and 10% yields respectively.

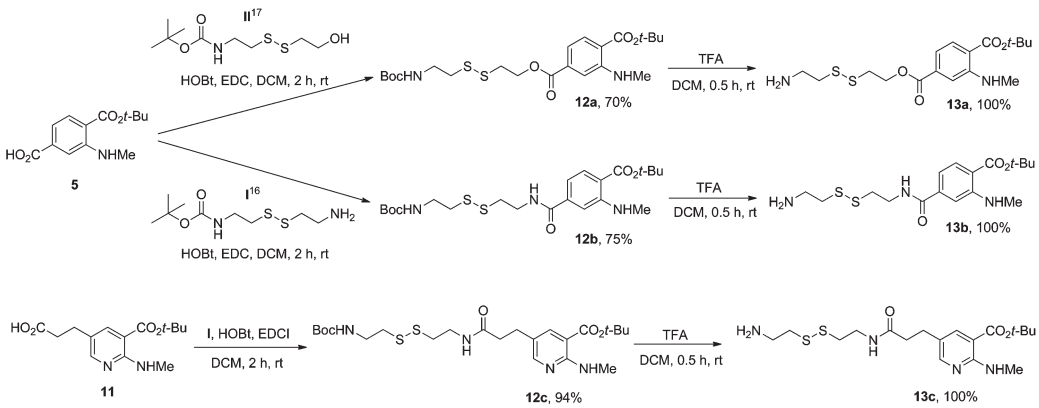
We first evaluated the IA derivatives **15a–15f** with synthetic nucleic acids, a 27-nt oligoribonucleotide and a 27-nt oligodesoxyribonucleotide, using the same procedure described above



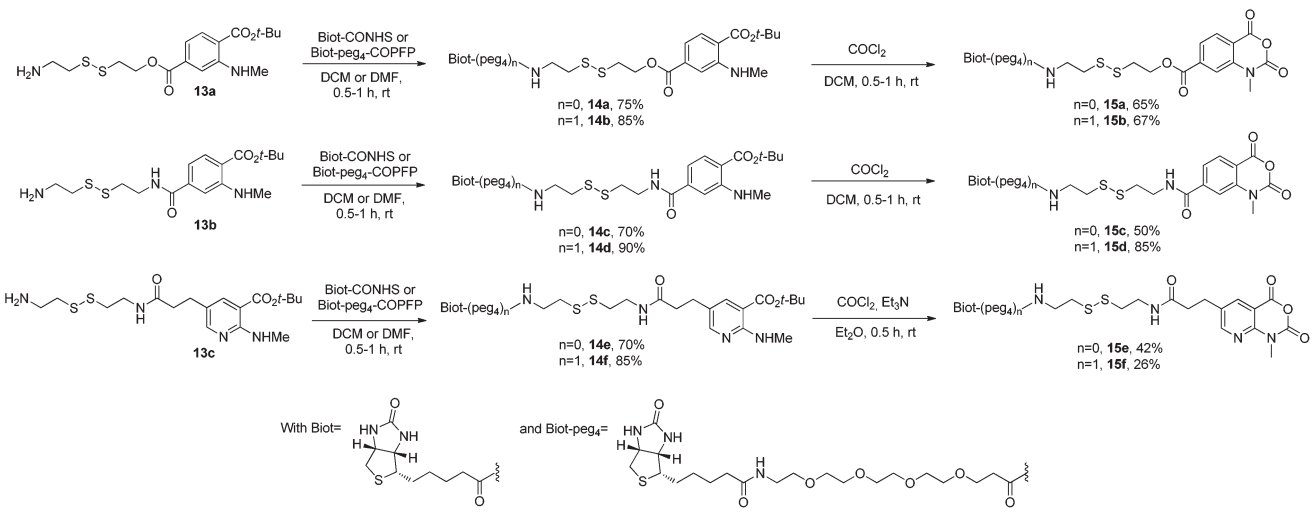
Scheme 2 Synthesis of *t*-butylaminoester **5** (isolated yields).



Scheme 3 Synthesis of *t*-butylaminoester **11** (isolated yields).



Scheme 4 Synthesis of cystamine derivatives **13a–c** (isolated yields).



Scheme 5 Synthesis of RNA tags **15a–f** (isolated yields).

for the screening of compounds **3a–i**. The percentages of tagged nucleosides were determined respectively at 65 °C and at room temperature and compared to the results obtained for the compound of first generation **A**. The selectivity of the acylation reaction was determined as the ratio of tagged nucleosides with both oligonucleotides (Fig. 4). At 65 °C, the percentage of tagged nucleosides for **A** was calculated to be 7.5% with RNA and 0.7% for DNA, giving a selectivity ratio of 10.7. The 7-carboxy and 8-azaisatoic anhydrides **15a–f** were found to be more reactive toward RNA with a tagging percentages ranging between 8.7 and 14.1%; the best results being obtained with aza compounds **15e** and **15f** with respective percentages of tagged nucleosides of 14.1 and 13.5%. These observations are in accordance with results previously obtained in the screening. Esters **15a–15b** and amides **15c–15d** exhibited the same reactivity, showing that the nature of the linkage on the carbonyl group has no influence. When comparing the results between pegylated and non-pegylated compounds no significant differences were observed in the acylation ratios. These results can be explained by the good

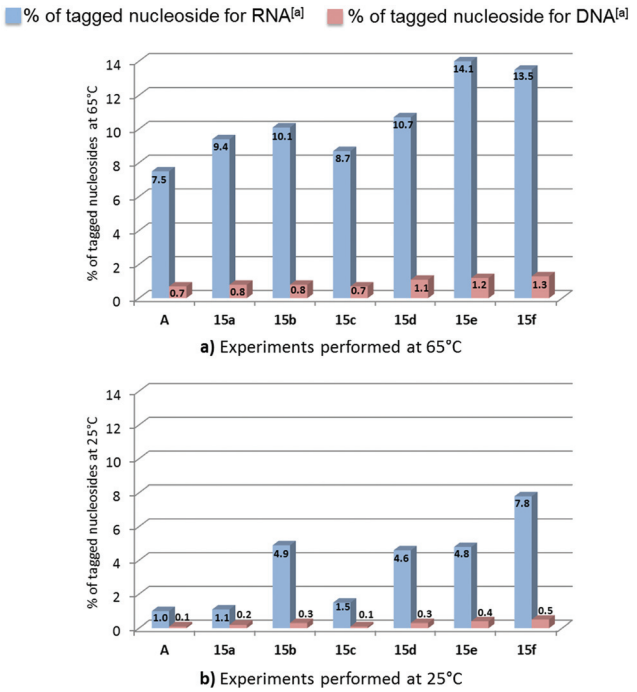


Fig. 4 Synthetic 27-nt RNA and 27-nt DNA (8 nmol) tagging with 30 mM of isatoic anhydride derivatives **15a–f** at 65 °C and 25 °C. [a]Ratio determined by LC/MS, see experimental data for details.†

solubility of all these compounds at 65 °C. No difference was observed in the selectivity between RNA and DNA acylation with the parent compound **A**. Indeed all the ratios were found to be between 10 and 12. We then turned our attention to the acylation reaction at room temperature. Compound **A** exhibited a very low reactivity with only 1.0% of RNA nucleosides acylated. For the carboxy derivatives, the esters and amides were found equally reactive, with the non-pegylated compounds **15a** and **15c** acylating 1% of RNA nucleosides, whereas the pegylated derivatives **15b** and **15d** were found to be quite more reactive than the parent compound **A**, underlining that both the intrinsic reactivity and the aqueous solubility of IA derivatives are two critical parameters to perform an efficient acylation of RNA nucleosides. These results were confirmed with aza-compounds **15e** and **15f** which were able to acylate RNA nucleosides in high ratios of 4.8 and 7.8% respectively. The non-pegylated aza-derivative **15e** was more reactive than the non-pegylated carboxy-compounds and as effective as the pegylated ones **15c** and **15e**. The pegylated aza-anhydride **15f** exhibited the best acylating properties, matching with its higher water solubility. The acylation ratio between RNA and DNA nucleosides was calculated to be around 15.5 for the three pegylated compounds **15b**, **15d** and **15f**, which represents an increase of selectivity of 50% compared to all experiments performed at 65 °C.

These preliminary results with oligonucleotides validated the interest in developing room-temperature RNA acylating reagents to gain a higher selectivity. It could be postulated that this higher selectivity for RNA *vs.* DNA at room temperature with more electrophilic isatoic anhydride derivatives would result from the combination of two effects. First, the lower reactivity of the 3′-OH group compared to the 2′-OH group as demonstrated by Weeks *et al.* with the 2′-deoxyATP being 10-fold less reactive than 3′-deoxyATP[18] would lead to a kinetic selectivity favoring thus acylation of the more reactive 2′-OH group of RNA. Secondly, the higher susceptibility of the more electrophilic isatoic derivatives to hydrolysis in the reaction medium[11] would lead to the rapid inactivation of their acylating properties, limiting thus the acylation of the less reactive 3′-OH of DNA.

We then selected the more reactive azaisatoic anhydride **15f** for further evaluation in the RNA extraction process using HIV RNA transcripts and calf genomic DNA as biological nucleic acid models.

The RNA extraction process was first applied to HIV RNA transcript and compared to calf genomic DNA. The separation efficiency was then studied using a mixture of the two nucleic acids. Nucleic acids were incubated with **A** or **15f** at two concentrations (1.5 and 30 mM). After removing the tag excess using magnetic silica particles, biotinylated nucleic acids were captured with streptavidin-coated magnetic beads and eluted after disulfide cleavage with dithiothreitol (DTT). The eluted nucleic acids were then quantified by fluorescence and extraction yields were determined for both RNA and DNA. The selectivity of the capture was evaluated by calculating the ratio of RNA/DNA extraction yields (Fig. 5). The control experiment performed with HIV RNA transcripts and calf genomic DNA without tag led to extracted yields below the detection limit. Non-specific interactions between streptavidin magnetic beads and nucleic acids appeared to be negligible. The compound of first generation **A** was evaluated at room temperature and gave significant extraction yields only using high concentration of tag (22% of extracted RNA at 30 mM), whereas using 1.5 mM only led to very low extraction yields (<5%). These results are consistent with the low reactivity observed for **A** toward an RNA oligomer at room temperature. At 65 °C this derivative was more efficient allowing RNA extraction with the lower tag concentration (27% of extracted RNA at 1.5 mM). The use of higher tag concentration led to an increase in the yield (66% at 30 mM) but in this case a six-fold decrease of selectivity was observed (extracted RNA/DNA ratio: 57 at 1.5 mM *vs.* 10 at 30 mM). With the 8-azaisatoic derivative **15f** RNA extraction yield of 24% was obtained at the lower concentration of 1.5 mM. At this low concentration, **15f** appeared to be as efficient at room temperature as **A** at 65 °C. Additionally, a clear increase of selectivity was observed when using azaisatoic **15f** at room temperature (extracted RNA/DNA ratio of 158 at 1.5 mM) compared to **A** at 65 °C (extracted RNA/DNA ratio of 57 at 1.5 mM) with similar RNA extraction yields. Interestingly increasing the temperature of the acylation step at 65 °C with **15f** at the lower concentration of 1.5 mM did not result in improved RNA extraction yields (21% at 65 °C *vs.* 24% at 25 °C), but only to a two-fold decrease of the selectivity ratio (extracted RNA/DNA ratio of 79 at 65 °C *vs.* 158 at 25 °C). At higher concentration of **15f** (30 mM), the extracted RNA yield was significantly increased at both room temperature and 65 °C (62% and 57%, respectively).

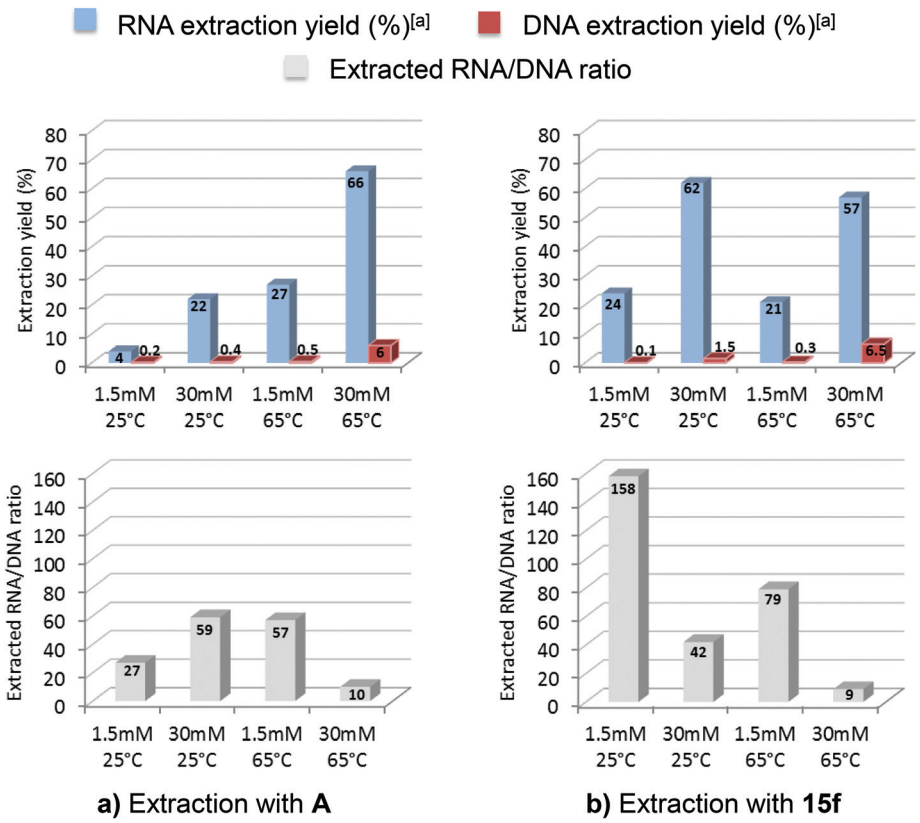


Fig. 5 Extraction of biological NA using **A** and **15f**. [a]Extraction yields determined using fluorescence spectroscopy, see experimental data for details.†

Unfortunately as previously observed with **A**, a decrease in the selectivity ratio was observed (42 at 25 °C, 9 at 65 °C). Taken together these results validate the hypothesis that secondary reactions with DNA can be minimized using acylating agents able to react under milder conditions.

Compound **15f** was further evaluated for its ability to separate the two nucleic acids starting from a mixture of RNA–DNA containing 10% of RNA and 90% of DNA in order to consider the predominance of DNA in biological samples. The acylation step was performed with the lower tag concentration of 1.5 mM at room temperature and compared to the parent compound **A** at 65 °C. The selectivity of the separation process was evaluated by spectrofluorescence quantification of the two nucleic acids after capture and elution (Table 2). At 65 °C, the use of isatoic anhydride **A** reversed the ratio of the nucleic acids with a resulting mixture containing 86% of RNA after the extraction process leading to a selectivity of 5. Performing the acylation step at room temperature with 8-azaisatoic derivative **15f** increased the selectivity of the process to 28 by lowering the quantity of extracted DNA. A strong RNA enrichment was achieved with **15f** and the RNA/DNA ratio reached 95/5 starting from an initial mixture containing 90% of DNA. Nevertheless, when we compared the selectivity obtained for the mixture of nucleic acids with the selectivity obtained when RNA and DNA are extracted separately, a strong decrease of selectivity was observed (28 *vs.* 158). This decreasing selectivity was probably due to interactions between the captured RNA and non acylated genomic DNA but not to enhancement of the DNA acylation.

To prove this hypothesis, the RNA HIV transcript was acylated using derivative **15f** at 30 mM. After removing the tag

Table 2 RNA extraction from biological RNA–DNA mixture using **A** and **15f**

Tag at 1.5 mM	Temperature (°C)	RNA/DNA initial ratio[a]	RNA/DNA final ratio[a]	RNA/DNA Selectivity[b]
A	65	10/90	86/14 ± 1	5 ± 0.9
15f	25	10/90	95/5 ± 1	28 ± 1.7

[a] Determined using fluorescence spectroscopy (n = 2), see experimental data for details. [b] Ratio determined from RNA and DNA extraction yields.

Table 3 Co-extraction of non-acylated DNA and captured RNA

Tag at 30 mM	Temperature (°C)	% of RNA extracted[a]	% of DNA recovered[a]
15f	25	42.0	2.1

[a] Determined using fluorescence spectroscopy, see experimental data for details.

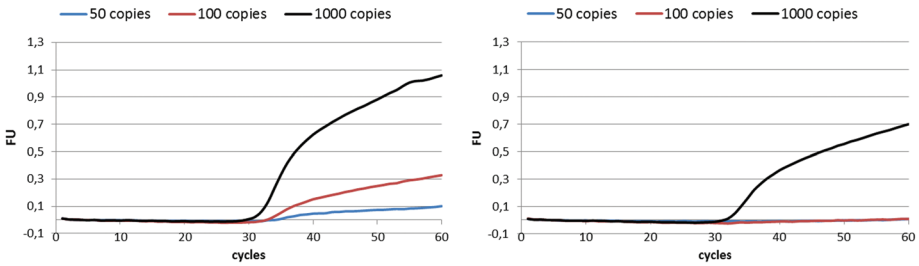


Fig. 6 RT-PCR of transcript HIV extracted with azaisatoic anhydride derivative **15f** (for control experiments using non-acylated RNA and electrophoresis of RT-PCR amplicons, see ESI†).

excess, genomic DNA was added to the mixture before the capture step with streptavidin magnetic beads. After DTT treatment and elution, RNA and DNA were quantified by fluorescence. As we had previously checked the absence of non-specific interaction between magnetic beads and nucleic acid, no significant amounts of DNA should be detected. In contrast, 2.1% of the initial quantity of DNA was recovered after the extraction process, demonstrating clearly that the presence of residual DNA was due to co-elution of non-acylated DNA with captured RNA and not to DNA acylation (Table 3).

As the amplification of RNA by RT-PCR remains a prerequisite for detection of low RNA concentrations in molecular biology and notably for diagnostic applications, we had previously shown that RNA captured by our method with compound **A** could be amplified even with low concentration of extracted RNA (50 copies).[10] We then evaluated the RT-PCR amplification of RNA obtained with compound **15f**. RNA HIV transcripts were first acylated by **15f** at 15 mM, and after capture and elution, RNAs were diluted to respectively 50, 100 and 1000 copies before performing the RT-PCR amplification (Fig. 6a). In accordance with previous results obtained with compound **A**, RNA amplification, after capture with **15f**, led to the positive HIV transcript detection, even with the lower concentration of 50 copies. In order to evaluate the limit of the extraction method, we performed the same experiment using **15f** at a high concentration of 180 mM. In this case, a positive detection was only obtained with high RNA concentrations of 1000 copies (Fig. 6b). It can be postulated that using a high concentration of tag increased the probability of acylation inside the RT-target sequence, stopping then the reverse transcriptase on 2′-*O*-adducts as reported by Weeks in the SHAPE protocol.[18] Nevertheless, in the extraction process, we used a low concentration of tag (1.5 mM) at which no inhibition of the amplification process should be observed.

Conclusions

In conclusion, we have designed new 2′-OH selective RNA-acylating agents based on isatoic anhydride structures and evaluated their uses for the extraction and separation of RNA from DNA. The modulation of the aromatic ring by introducing electron withdrawing groups or using a pyridine analog of isatoic anhydride, led to an increase in the reactivity of these compounds toward RNA and allow the efficient capture and recovery of RNA at room temperature, when the compound of first generation **A** was only efficient at elevated temperatures. Due to its higher water solubility, the pyridine derivative **15f** was selected and was able to extract RNA starting from a RNA–DNA mixture with a higher selectivity than the previous compound **A**. The residual quantity of DNA detected after the extraction process, was mainly due to interaction of non-acylated DNA with captured RNA and not to DNA acylation. Moreover, we have confirmed the compatibility of this extraction technique with the amplification of RNA for detection applications. Besides its use for the selective extraction of RNA, this new room-temperature efficient RNA-acylating agent could find wider applications in the field of bioconjugate chemistry or chemical biology.

Acknowledgements

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