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Rapid synthetic access to caged RNA

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Light-activatable RNA with "caged" nucleobases is a versatile tool for light-control of RNA interference, riboswitch folding or miRNA or ribozyme action. Till date the respective caged and protected phosphoramidites for the solid-phase RNA synthesis are obtained in lengthy syntheses. Herein we explore the use of convertible nucleoside residues for a rapid access to nucleobase-caged RNA. In these studies we have also prepared the first RNA with a caging group on an N^2-position of a guanosine. The obtained caged sequences are compared in their duplex destabilizing ability via melting temperature measurements.

Keywords: RNA, Caged RNA, Light-activatable RNA, Duplex destabilizing

The regulation of a molecular process with light is a very powerful and precise way to conduct an experiment[1-5]. Light is an ideal trigger signal because it can be very selective if there are no other light-responsive elements in the system – which is often the case. If the appropriate wavelength is used it can be harmless and many samples, setups or model organisms are transparent. Furthermore, the technical access is also very well established. For example a confocal laser scanning microscope has already all the elements that are needed to conduct such an experiment; a wide range of laser light sources exist which can be routed into such a microscope. The software of such microscopes allows defining arbitrary regions of interest which are then scanned with the laser light under careful control of intensity. If the technology which transduces the laser signal into a biological effect has been previously installed in the sample of interest, experiments are possible in which non-equilibrium conditions ("signal cues") can be generated under sophisticated control of area, timing and extent of the effect.

One such technology which couples external light signals to an internal effect is the "caging" technology[1,2] in which a compound that can trigger an interesting effect is temporarily inactivated with a photolabile protecting group. This principle has been first realized around the year 1977[6-8] and has been extended to nucleic acids only much later. Nucleic acids can be "caged" for example by attaching photolabile "caging groups" on the phosphate backbones, on the 2-OH groups in the case of RNA, via the use of photolabile linking residues-either nucleosidic or non-nucleosidic-or via blocking the Watson-Crick base pairing capabilities of the nucleobases[1,9]. We focus primarily on the latter approach. These residues can be regarded as temporary mismatches which can be rescued with light and have already been used in a large number of applications including regulation of gene expression and aptamer function[1,9]. They have also, for example, been elegantly used by Schwalbe ***et al***.[10] to trigger folding events ***in situ*** in time-resolved NMR experiments.

In this toolbox of residues it is desirable to use a range of different caging groups because this allows the interesting prospect of selectively addressing individual caging groups, for example, on different molecules using light of different wavelengths. First examples of this principle have already been realized[11,12]. However, especially in the case of caged RNA, this attempt is very tedious since the synthesis of each residue is rather long and requires the separation of 2′/3′ regioisomers which can in some cases only be performed using MPLC methods. On the other hand, caged RNA is a very useful tool for studying aspects of translation, riboswitch folding and action as well as RNA interference, miRNA and ribozyme action. In the present study, we have explored whether a new rapid access to caged RNA is possible using convertible nucleosides.

The convertible nucleoside strategy has been originally developed by Verdine ***et al***.[13,14] and has recently been used by Höbartner ***et al***.[15] to introduce paramagnetic spin labels into RNA. Currently, residues such as the ones shown in Fig. 1 are commercially available. They are all characterized by a reactive group (marked in gray) which survives the solid phase synthesis conditions. After the completion of the last coupling step these reactive groups can be exchanged with different nucleophiles. Then the RNA is cleaved and processed to yield the final product. In our case this would mean that generic precursors of caged RNA could be synthesized using only commercially available residues. Thus, the synthesis effort would be dramatically reduced to only the synthesis of the nucleophilic caging group reagents. Only after the last step of the RNA synthesis the caging group of choice would be installed from this common precursor.

Materials and Methods

The caging group reagents, NPE-NH_2, NPP-OH and NPP-NH_2, were synthesized as previously described[16-18]. The convertible nucleoside residues used in this study were purchased from Berry & Associates, Dexter, USA and introduced into the RNA sequence 5′-GCAUAAAXAAAGGUG-3′ using TBDMS-protected phosphoramidites for the other residues. For the solid phase synthesis, an ABI 392 synthesizer was used with the standard RNA coupling protocol. The respective counter strands were synthesized analogously.

For the subsequent test modification with the caging group reagents, a tip of a spatula of the solid phase material was mixed with 30 µL of a 2.5 ***M*** solution of the caging group reagent and the suspension was shaken for the given amount of time. Then, where applicable (see Table 1), 100 µL of aqueous ammonia (32%) or $MeNH_2$ in MeOH (33%) was added and the suspension was further shaken at room temperature for 40 h or overnight, respectively. After that, the suspension was filtered and the solvent of the filtrate was removed under vacuum. For the 2′-OH deprotection, the residue was treated with a mixture of ***N***-methylpyrrolidone (NMP), triethylamine (TEA) and triethylamine trihydrofluoride (TEA•3HF) (6:3:4, 100 µL, 42 °C, 2 h). The product

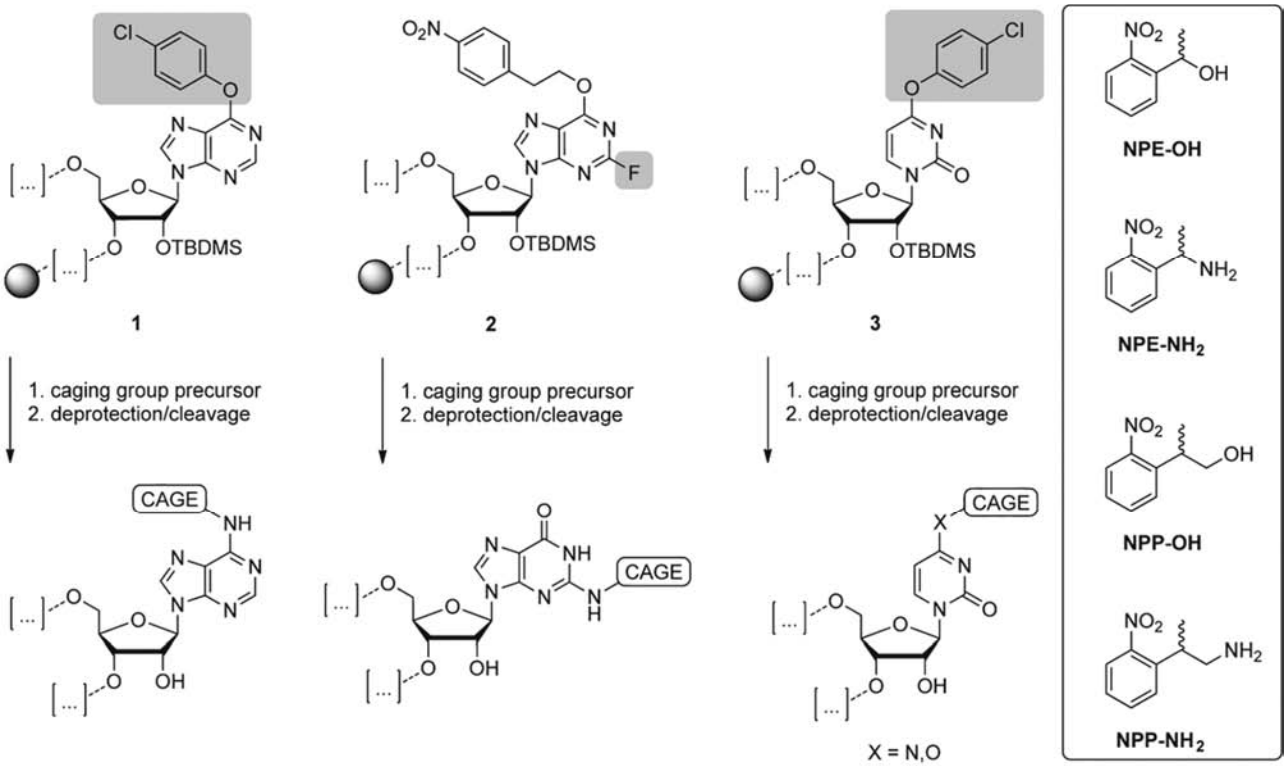

Fig. 1 — Overview of the convertible nucleoside strategy applied in this study for the rapid access to caged RNA. The three residues shown on top are commercially available and can be used for solid phase synthesis. After the last coupling step, the reactive group marked in gray can be replaced in a nucleophilic attack with a suitable caging group reagent. The reagents used in this study are shown in the box on the right.

Table 1 — Results of substitution studies with the RNA sequences, GCA UAA AXA AAG GUG, where X is one of the indicated convertible nucleosides shown in Fig. 1

Convertible nucleoside	Nucleophile	Solvent	Incubation time (d)	Temp. (°C)	Cleavage cond.	Result
1	NH_3	H_2O	1	r. t.	-	+
1	$MeNH_2$	EtOH	1	r. t.	-	+
1	NPE-NH_2	MeCN	6	60	$MeNH_2$	-
1	NPP-NH_2	MeOH	2	r. t.	-	+
1	NPP-OH	MeCN	7	42	NH_3	-
2	NH_3	H_2O	1	r. t.	-	+
2	$MeNH_2$	EtOH	1	r. t.	-	+
2	NPE-NH_2	MeCN	2	42	$MeNH_2$	+
2	NPP-NH_2	MeOH	2	42	-	+
3	NH_3	H_2O	1	r. t.	-	+
3	NPE-NH_2	MeCN	5	60	$MeNH_2$	+
3	NPP-NH_2	MeOH	1	42	-	+
3	NPP-NH_2	MeOH	2	r. t.	-	+
3	MeOH	-	7	42	NH_3	-
3	NPP-OH	MeCN	7	42	NH_3	-

was first purified using NAP-5 columns. Then the solvent was evaporated and the crude product was purified via anion exchange HPLC (Dionex, BIO LC, DNA Pac, 4×250 mm, buffer A: 25 m***M*** Tris buffer ***p***H 7, buffer B: buffer A + 1 ***M*** LiCl) followed by a second run using RP-HPLC (Nucleosil 100−5 C18, buffer A: 0.1 ***M*** triethylammonium acetate ***p***H 7, buffer B: acetonitrile). The identity of the oligonucleotides was established by ESI-MS (Table 2).

Melting temperatures of the (caged) RNA strands and their respective counter strands were determined in analogy to previously published procedures[17].

Results and Discussion

Three convertible RNA nucleoside residues were investigated in this study, which were also the ones presented in the original paper by Verdine ***et al***.[14] After an attack with amine nucleophiles, the left residue in Fig. 1 yields modified adenosine residues with caging groups on the N^6-position. Similarly, the middle residue in Fig. 1 yields N^2-caged guanosines. It is important to note that primarily up to now O^6-caged guanosine residues are the ones which have been investigated in both the DNA and RNA series. Hence, the present approach also gives rapid access to a presently unknown caged RNA residue. Finally, the convertible nucleoside on the right in Fig. 1 is at least potentially a common precursor for both N^4-modified cytidine and O^4-modified uridine residues depending on whether an N- or an O-nucleophile is used for the attack, respectively. Hence, with this set of three commercially available building blocks all four RNA residues are potentially accessible in a caged form.

Table 2 — Characterization of the synthesized caged RNA sequences, GCA UAA AXA AAG GUG, where X is the indicated residue

X	Mol. wt. (g/mol)	
	Calc.	Measured
$A^{N6\text{-}NH2}$	4867.7	4867.7
$A^{N6\text{-}NHMe}$	4881.7	4881.7
$A^{N6\text{-}NPP}$	5030.9	5030.8
$G^{N2\text{-}NH2}$	4883.7	4883.8
$G^{N2\text{-}NHMe}$	4897.7	4897.8
$G^{N2\text{-}NPE}$	5033.0	5032.9
$G^{N2\text{-}NPP}$	5046.7	5046.9
$C^{N4\text{-}NH2}$	4843.7	4843.8
$C^{N4\text{-}NPE}$	4992.9	4992.8
$C^{N4\text{-}NPP}$	5006.9	5006.8

For the present studies we chose a test sequence which was the RNA analogue of the benchmark sequence which had been used for a previous systematic study of caged residues in the DNA series[18].

For the modification, the conditions of Table 1 were applied. Treatment of the solid support with the sequence containing residue (**1**) with ammonia or methyl amine (1 d at r.t.) readily yielded the respective oligonucleotides for comparison after fluoride deprotection. Similarly, a treatment with NPP-NH_2 in methanol yielded the NPP-caged RNA sequence with only a slight increase in reaction time (2 d at r.t.). The nucleophilicity of this caging group reagent was also sufficient to cleave the sequence off the support and to remove all the protecting groups. However, an analogous reaction was not possible using the more sterically crowded NPE-NH_2 instead. We tried conditions such as 60 °C for 6 d

(with a solution of NPE-NH_2 in MeCN or DMSO). We also had to add $MeNH_2$ in the end for the final cleavage since NPE-NH_2 itself is not nucleophilic enough to effect both the deprotection of the nucleobases and the phosphodiester backbone and the cleavage from the solid support. However, only the methyl amine adduct at the position of the convertible residue (**1**) could be recovered (or – depending on reaction time – a soluble and otherwise deprotected intermediate with the 4-chlorophenyl-substitutent still in place). Höbartner ***et al***.[15] had also problems in installing a similarly sterically demanding spin label on an adenosine using a TEMPO-NH_2. However, unlike herein, they succeeded in the end – albeit with little yield[15]. In the test series using the convertible nucleoside (**2**) again the reference modifications with ammonia and methyl amine proceeded cleanly. This time both the sequence with the previously unknown NPE- and the NPP-caged residue could be obtained with relatively mild conditions (2 d, 42 °C). However, again in the case of NPE-NH_2, an extra treatment step with methyl amine had to be added after the incubation step with the caging group reagent. The sequences containing the common precursor (**3**) were first treated with nitrogen nucleophiles. A control reaction with ammonia again proceeded as expected. In this series a reaction with NPE-NH_2 was again possible, but an incubation time of 5 d at 60 °C was necessary, as well as the successive methyl amine cleavage step, yielding a sequence with an N^4-caged cytidine residue. The modification with NPP-NH_2 proceeded under milder conditions because either incubation for 1 d at 42 °C or for 2 d at room temperature was sufficient and a subsequent extra cleavage step with methyl amine was not necessary. Finally, we tried also to obtain O^4-caged uridine-containing sequences. Therefore, we treated the solid phase material at first with methanol for 7 d at 42 °C. However, we could not obtain the desired modified product sequence. Addition of base such as triethylamine or diisopropylethylamine did not change this picture, while addition of DBU lead to a degradation of the RNA. Subsequent treatment with ammonia only afforded the respective cytidine sequence. Consequently, a reaction with NPP-OH was also not possible under the applied conditions. Also, a reaction with residue (**1**) which would have yielded an O^6-caged inosine residue was not possible under our conditions. Similar difficulties with oxygen nucleophiles in convertible nucleophile approaches have been observed before[19].

Then we used the obtained sequences in this investigation to continue our systematic evaluation of the performance of caged residues in the destabilization of duplex structures. We have previously published a survey in the DNA series[18]. The results in the present RNA series are shown in Table 3. First of all, the unmodified RNA versions of our benchmark sequences generally melt at higher temperatures compared to their DNA counterparts under the same conditions which is in accord with established tendencies[20]. For example, the sequence with X = A melts at 54.8 °C in the RNA series (Table 3) and at 49.8 °C in the DNA series[18] (and similarly for X = G with 59.5 °C in the RNA and 51.9 °C in the DNA series and for X = C with 58.9 °C in the RNA and 54.3 °C in the DNA series.). Substitution of a central adenosine with $A^{N6\text{-}NPP}$ affords destabilization with a 4.9 °C reduction in the melting temperature. In the DNA series, a reduction of 10.5 °C was observed[18]. Apparently, an RNA-RNA duplex is more difficult to destabilize with caging groups. The situation with $G^{N2\text{-}NPE}$ and $G^{N2\text{-}NPP}$ in the central position is more difficult to compare because no similar residues exist yet in the DNA series. However, the melting temperature reduction with the new $G^{N2\text{-}NPE}$ and $G^{N2\text{-}NPP}$ residues is in the same order of magnitude as the one with $A^{N6\text{-}NPP}$. A similar behavior was found with $dA^{N6\text{-}NPP}$ and $dG^{O6\text{-}NPP}$ in the DNA series[18]. The residues, $C^{N4\text{-}NPE}$ and $C^{N4\text{-}NPP}$, finally showed an unexpectedly high reduction in melting temperature of 8.0 °C and 7.8 °C, respectively. In the DNA series, we had obtained values of 8.0 °C and 12.7 °C (Ref. 18).

Table 3 — Results of melting temperature determinations using the sequences, GCA UAA AXA AAG GUG, where X is the indicated residue with the respective matching RNA counter strand

X	T_m (°C)	ΔT_m (°C)
A	54.8±0.1	-
$A^{N6\text{-}NPP}$	49.9±0.4	4.9
G	59.5±0.1	-
$G^{N2\text{-}NPE}$	55.8±0.4	3.7
$G^{N2\text{-}NPP}$	54.8±0.2	4.7
C	58.9±0.6	-
$C^{N4\text{-}NPE}$	50.9±0.3	8.0
$C^{N4\text{-}NPP}$	51.1±0.3	7.8

Conclusions

In summary, we have tested the original Verdine RNA convertible nucleoside residues for rapid access to caged RNA and found that NPP caging groups could be installed easily on A, G and C residues. An NPP caging group is preferred over an NPE caging group since it does not form potentially harmful nitroso species upon uncaging as does an NPE caging group. The introduction of NPE caging groups was more difficult and was only possible in the case of G and C with an extra successive nucleophilic treatment. The introduction of oxygen nucleophiles such as for an O^4-caged U residue was not successful in our hands. We also synthesized for the first time an RNA sequence with a N^2-caged guanosine residue. In subsequent melting temperature studies we have extended our systematic survey of the duplex destabilization with nucleobase-caged residues to RNA-RNA sequences. These findings can guide new experiments in which the now rapidly accessible caged RNA is used in functional studies.

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