

Synthesis of oligo-RNAs with photocaged adenosine 2'-hydroxyls

Steven G Chaulk & Andrew M MacMillan

Department of Biochemistry, University of Alberta Edmonton, Alberta, Canada T6G 2H7. Correspondence should be addressed to A.M.M. (andrew.macmillan@ualberta.ca).

Published online 26 April 2007; doi:10.1038/nprot.2007.154

This protocol describes a general method for the preparation of RNAs in which the reactivity or hydrogen-bonding properties of the molecule are modified in a photoreversible fashion by use of a caging strategy. A single caged adenosine, modified at the 2' position as a nitro-benzyl ether, can be incorporated into short RNAs by chemical synthesis or into long RNAs by a combination of chemical and enzymatic synthesis. The modified RNAs can be uncaged by photolysis under a variety of conditions including the use of a laser or xenon lamp, and the course of this uncaging reaction may be readily followed by HPLC or thin-layer chromatography.

INTRODUCTION

RNA molecules, in addition to being carriers of genetic information, play key roles in the regulation of transcription and translation and form the catalytic core of both the spliceosome and ribosome. The study of many RNA systems is complicated by the dynamic nature of RNA secondary and tertiary structures, the transient nature of RNA · RNA or RNA · protein complexes, and in some cases the chemical reactivity of the RNA itself.

One useful method to study discrete RNA (and DNA) structures has been to limit available conformations through site-specific intrastrand crosslinking¹. An alternative approach, widely used in the study of biomolecular recognition or reactivity, is the “caging” of the molecule of interest whether it be a substrate, cofactor or enzyme². Classically, caged molecules are chemically modified such that they become activated upon photolysis. In the context of an RNA molecule, a caging approach might be used to block either chemical reactivity of the RNA or formation of secondary or tertiary structure (Fig. 1). The caged RNA system can be studied both before and after photolysis, thus permitting characterization of the two states and the transition between them.

The first application of the caging approach to studies of nucleic acid structure and function involved blocking the chemical reactivity associated with an RNA functionality, the 2'-hydroxyl group^{3–5}, as specific RNA 2'-hydroxyls act as nucleophiles in a number of biologically important transesterifications^{6–11}. In addition, interactions with 2'-hydroxyl groups are critical in the formation of higher order RNA structures as well as protein · RNA complexes.

Caging of a single 2'-hydroxyl in a short synthetic oligonucleotide blocks the cleavage reaction catalyzed by the hammerhead ribozyme⁶; cleavage is initiated by photolysis of the ribozyme · substrate complex³. This approach has also been extended to the caging of the branch adenosine¹¹ in a full-length precursor mRNA (pre-mRNA) for studies of RNA processing by the mamma-

lian spliceosome^{4,5}. Caging effectively isolates spliceosome assembly from catalysis of the splicing transesterifications, permitting a closer examination of the mechanisms of each. Thus, it has been established that ATP hydrolysis, while required for splicing, is not directly coupled to the first chemical step and also that phosphatase activities implicated in promoting both steps of splicing occur before the first chemical step^{4,5}.

Subsequent to our initial report³, the site-specific caging of nucleic acids has been widely applied to regulate a variety of activities or processes including transcription, aptamer and DNA-enzyme activity, DNA replication and the formation of higher order RNA structures^{12–19}. These experiments have involved either direct modification of pyrimidine or purine functionalities or of groups appended to them. Nonspecific caging of the phosphodiester backbone has been used to regulate gene expression in zebrafish embryos and to modulate siRNA activity in HeLa cells^{20,21}.

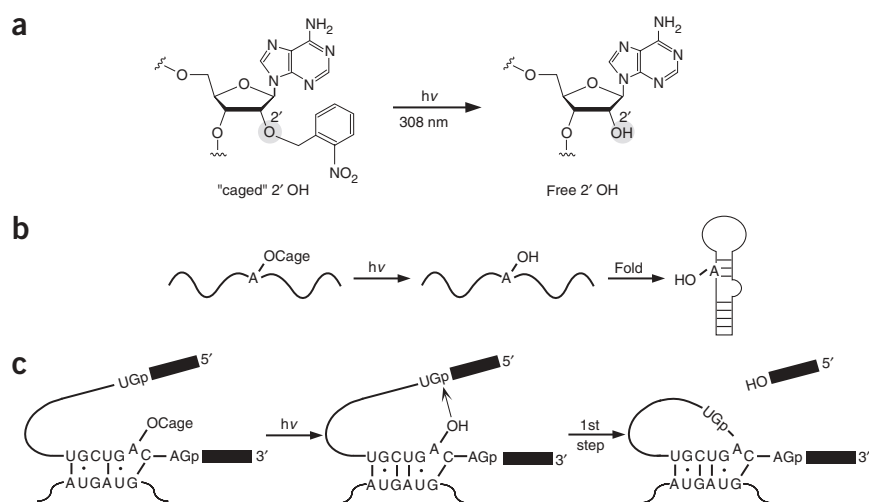


Figure 1 | Caged 2'-adenosines as tools for the study of RNA structure and function. (a) Caging of the 2'-hydroxyl of adenosine within RNA with an *o*-nitrobenzyl group that can be removed by photolysis; (b) caging the 2'-hydroxyl as a method of blocking structure formation for the examination of RNA folding pathways; (c) caging the 2'-hydroxyl as a method of blocking reactivity for the examination of reactions involving RNA such as pre-mRNA splicing.



Our approach to caging the 2'-hydroxyl functionality relies on earlier work in which the development of methods for the automated synthesis of RNA molecules necessitated strategies for protection of the reactive 2'-hydroxyl functionality. A variety of solutions to this problem have been explored including the use of the photo-labile *o*-nitrobenzyl moiety as a protecting group for the 2'-hydroxyl of the four RNA nucleosides^{22–24}. The synthesis of a caged adenosine phosphoramidite is straightforward (Fig. 2) and is especially facilitated by the fact that the desired 2'-modified adenosine nucleoside can be precipitated from the reaction solution. The caged adenosine is efficiently incorporated by standard automated synthesis into short synthetic oligomers that can then be purified by a combination of gel electrophoresis and HPLC (Fig. 3). The presence of the modified nucleotide is readily confirmed by enzymatic digestion and HPLC analysis (Fig. 4). This analysis also establishes that uncaging of the RNA proceeds with minimal if any damage to the nucleic acid.

This protocol describes the synthesis and characterization of a short RNA sequence in which a reactive 2'-hydroxyl is masked in a hammerhead ribozyme substrate (Steps 16–23). Further to this, a single caged nucleotide can be incorporated into a long RNA by

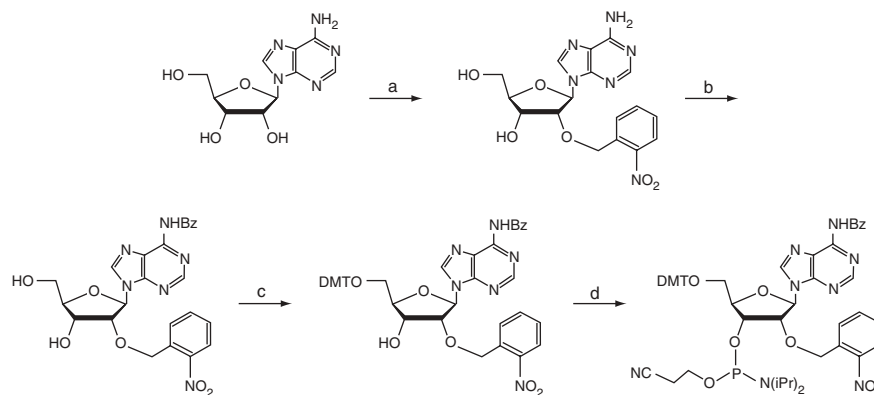


Figure 2 | Synthesis of 2'-caged adenosine phosphoramidite. a: *o*-nitro-BnBr, NaH, DMF; b: trimethylsilylchloride, benzoyl chloride, pyr; c: DMTCl, DMAP, pyr; d: cyanoethyl diisopropylaminochlorophosphite, *i*Pr₂EtN, THF.

joining a synthetic oligomer containing the caged residue (prepared as in Steps 16–23) to T7 RNA polymerase transcripts using the Moore–Sharp ligation protocol (Fig. 5) (see refs. 25,26) This protocol describes the synthesis and characterization of one such RNA: a model human pre-mRNA splicing substrate (Steps 24–30).

The 2'-caged RNA is activated by photolysis using either a laser or a powerful lamp (depending on the application: see below), and the course of photolysis is readily monitored either by HPLC or thin-layer chromatography (TLC) analyses of enzymatic digests of the oligonucleotide (Figs. 3 and 4).

MATERIALS

REAGENTS

- Solvents for organic synthesis are dried by conventional means or may be purchased commercially (Aldrich; Sure/Seal)
- Anhydrous pyridine (Aldrich, cat. no. 270970-100ML)
- Anhydrous dimethylformamide (DMF; Aldrich, cat. no. 227056-100ML)
- Anhydrous THF (Aldrich, cat. no. 401757-100ML)
- Flash silica gel 40–63 μm (Silicycle, cat. no. R10030B)
- TLC plates: silica gel 60 F254 2.5 × 7.5 cm (EM Merck, cat. no. EM-15327-1)

- Adenosine (Aldrich, cat. no. 14,659-5)
- Sodium hydride (Aldrich, cat. no. 45,291-2)
- 2-Nitrobenzylbromide (Aldrich, cat. no. 10,779-4)
- Chlorotrimethylsilane (Aldrich, cat. no. C7,285-4)
- Benzoylchloride (Aldrich, cat. no. 25,995-0)
- Dimethylaminopyridine (DMAP; Aldrich, cat. no. 52,280-5)
- 4,4'-Dimethoxytrityl chloride (DMTCl; Aldrich, cat. no. 10,001-3)
- *N,N*-Diisopropylethylamine (Aldrich, cat. no. D12,580-6)
- 2-Cyanoethyl-*N,N*-diisopropylaminochlorophosphite (Aldrich, cat. no. 30,230-9)
- T7 RNA polymerase (Promega, cat. no. P 2075)
- 2'-FPMP RNA phosphoramidites (Rasayan Inc.)
- 500 Å FPMP-controlled pore glass resin (Rasayan Inc.)
- Methanolic ammonia (Aldrich, cat. no. 49,914-5)
- Calf intestinal alkaline phosphatase (NEB, cat. no. M0290S)
- Polynucleotide kinase (NEB, cat. no. M0201S)
- T4 DNA ligase (cat. no. P4557)
- Snake venom phosphodiesterase (AmershamBiosciences, cat. no. E202404)
- RNase A (Roche, cat. no. 109142)
- RNase T1 (Roche, cat. no. 109193)
- RNasin (Promega, cat. no. N2515)
- Cellulose PEI 4 × 10 cm TLC plates (J.T. Baker, cat. no. 4474-04)
- [γ -³²P]ATP (6,000 Ci mmol⁻¹, 5 mCi ml⁻¹; NEN, cat. no. NEG035C)
- C18 cartridge (Burdick & Jackson, cat. no. 9200)
- Urea (Aldrich, cat. no. U270-9)
- Trizma acid (Tris-HCl; Sigma, cat. no. T6666)
- Trizma base (Tris base; Sigma, cat. no. T6791)
- Boric acid (Sigma, cat. no. B7660)
- EDTA (Sigma, cat. no. E1644)
- Sodium acetate (Sigma, cat. no. S7545)
- Phenol (Sigma, cat. no. P4557)
- Chloroform (Sigma, cat. no. C2432)
- Isoamyl alcohol (Sigma, cat. no. I9392)
- HEPES (Sigma, cat. no. H7523)
- PVP-40 (Sigma, cat. no. PVP-40)

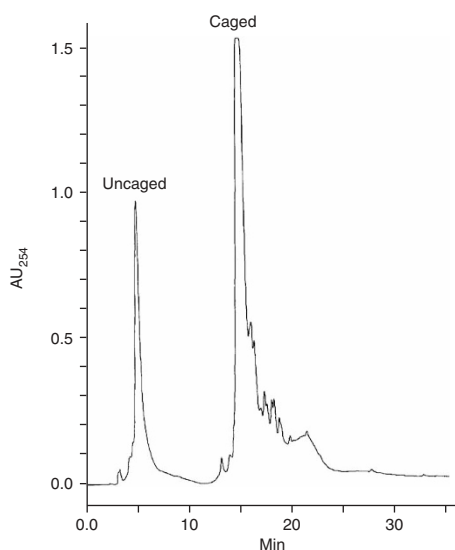


Figure 3 | HPLC purification of caged hammerhead substrate RNA. Following automated synthesis, deprotection and initial gel purification, uncaged RNA is separated from caged material by reverse-phase HPLC (elution times of ~5 and ~15 min, respectively, under the conditions of this experiment; see text).

PROTOCOL

Figure 4 | HPLC nucleoside composition analysis of caged and photolyzed hammerhead substrate RNA. (a) Enzymatic digestion of caged hammerhead substrate RNA 5'-GGGUGUA*UGGUU-3' (A*: 2'-caged adenosine; G: guanosine; U: uridine); (b) enzymatic digestion of hammerhead substrate RNA containing a single 2'-caged adenosine residue following photolysis with an excimer laser (A: adenosine; G: guanosine; U: uridine).

- Bromophenol blue (Sigma, cat. no. B8026)
- Xylene cyanole (Sigma, cat. no. X4126)

EQUIPMENT

- Standard equipment for organic synthesis including glassware, Teflon stir bars, septa, syringes, needles, magnetic stir plate, flash column (4 cm × 30 cm) and a vacuum pump
- Plastic syringes are appropriate for transfers of all solvents except THF
- Pyrex tubes 4–5 cm long (cut NMR tubes are suitable) with cap
- Lambda Physik EMG 201 MSC excimer laser or water-jacketed 1,000 W Oriol Xenon arc lamp
- Applied Biosystems 392 DNA/RNA Synthesizer
- UV spectrophotometer
- PAGE electrophoresis unit with power supply

PROCEDURE

Synthesis of caged phosphoramidite

Synthesis of 2'-O-(2-nitrobenzyl)adenosine ● TIMING 12 h

1| Dissolve adenosine (1.0 g, 3.75 mmol; evaporated using a rotary evaporator three times from dry pyridine to remove water) in 34 ml of hot dry DMF in a round-bottom flask containing a stir bar. To the stirring solution, add sodium hydride (225 mg, 60% (w/v) in oil, washed three times with hexanes) as a suspension in 4 ml of dry DMF using a 5 ml syringe. Stir the resulting solution at 0 °C for 45 min under nitrogen.

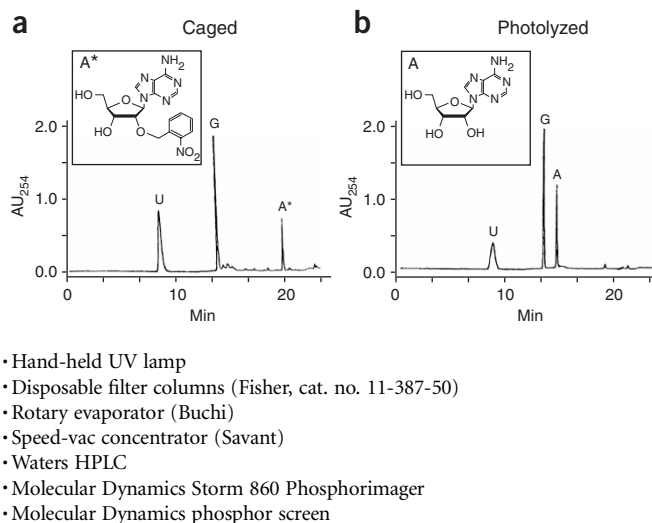
! CAUTION Sodium hydride is highly flammable and reacts violently with water.

2| Add 1.21 g (5.6 mmol) of 2-nitrobenzylbromide (*o*-nitro-BnBr) in 2 ml of dry DMF using a syringe. Stir the reaction mixture at room temperature (22 °C) under nitrogen for 5 h.

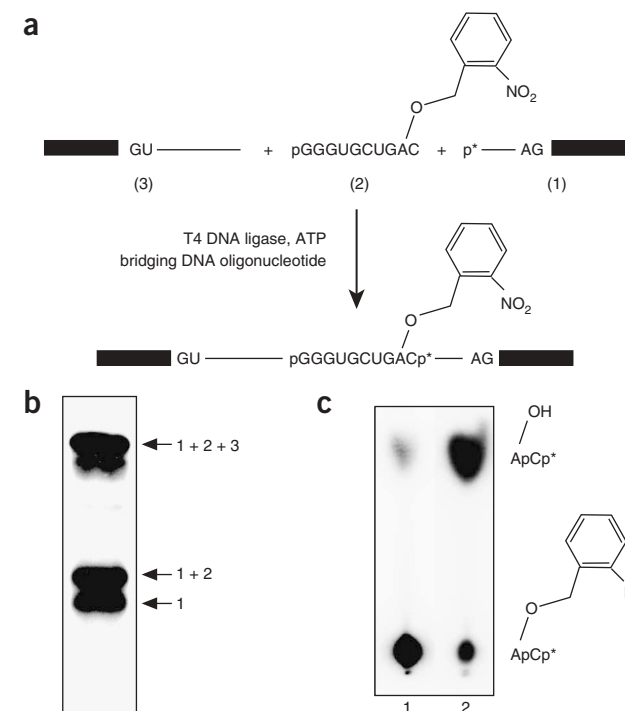
! CAUTION 2-nitrobenzylbromide is a lachrymator.

3| Pour the reaction into 375 ml of ice-cold water and stir the resulting mixture at room temperature.

■ **PAUSE POINT** The reaction thus quenched may be left stirring overnight.



- Hand-held UV lamp
- Disposable filter columns (Fisher, cat. no. 11-387-50)
- Rotary evaporator (Buchi)
- Speed-vac concentrator (Savant)
- Waters HPLC
- Molecular Dynamics Storm 860 Phosphorimager
- Molecular Dynamics phosphor screen



4| Collect the resulting yellow precipitate by vacuum filtration and dry *in vacuo*. The yield is assumed to be quantitative for the next step in the preparation.

▲ **CRITICAL STEP** Although the caged nucleoside is relatively stable in ambient light, at this stage and beyond, during prolonged reaction periods, the compound can be protected by wrapping glassware in an aluminum foil.

Synthesis of 2'-O-(2-nitrobenzyl)-N⁶-benzoyladenosine

● TIMING 12 h

5| Add 3.8 ml (30 mmol) of trimethylsilylchloride via syringe to a suspension of 2'-O-(2-nitrobenzyl)adenosine (1.5 g, 3.73 mmol) in 20 ml dry pyridine under nitrogen. Stir the mixture for 30 min at room temperature.

Figure 5 | Synthesis of pre-mRNAs containing a caged 2' hydroxyl at the branch position. (a) Schematic of ligation: a synthetic oligomer containing a single modified adenosine residue (2: 5'-GGGUGUCA*C-3'; nucleotides 146–156) was ligated to T7 RNA polymerase transcripts (1: nucleotides 157–234; 3: nucleotides 1–145) to yield full-length (234 nucleotide) caged PIP85.B pre-mRNA²⁶ containing a single ³²P radiolabel (p*); (b) denaturing PAGE purification of ligation (1: 3' transcript; 1 + 2: ligation of caged synthetic oligomer to 3' transcript; 1 + 2 + 3: full-length pre-mRNA); (c) TLC analysis of photolysis of caged pre-mRNA. Lane 1: unphotolyzed caged pre-mRNA digested with RNase A and RNase T1; lane 2: photolyzed uncaged pre-mRNA digested with RNase A and RNase T1.

- 6| Add 2.4 ml (20.6 mmol) of benzoyl chloride using a syringe and stir the reaction for another 2.5 h.
- 7| Cool the resulting mixture to 0 °C in an ice/water bath. Add 4 ml of water and stir the reaction for 5 min.
- 8| Add aqueous ammonia (33% (w/v), 8 ml) and continue stirring for an additional 30 min.
- 9| Concentrate the mixture *in vacuo* using a rotary evaporator and purify the product by silica gel flash chromatography using 5% (v/v) methanol/methylene chloride as eluant ($R_f = 0.3$) to yield 647 mg of 2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine (43% from adenosine).
 - ▲ **CRITICAL STEP** This chromatographic separation requires a "dry load" of the reaction product: add 5–6 ml of silica gel to the concentrating reaction mixture (Step 9) and pour the dried silica/reaction product mixture directly on top of the packed silica flash column. Pour the eluant directly on the dry powder and run the column normally.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine ● **TIMING 12 h**

10| Dissolve 455 mg (1.13 mmol) of 2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine (evaporated three times from dry pyridine using a rotary evaporator), 6 mg (0.05 mol%) of DMAP and 355 mg (1.1 mmol) of DMTCI in 2 ml of dry pyridine. Stir the reaction mixture at room temperature for 5 h under nitrogen.

11| Concentrate the resulting mixture *in vacuo* and purify the product by silica gel flash chromatography using ethyl acetate as eluant ($R_f = 0.75$) to yield 562 mg (74%) of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine. Note that trityl-containing compounds can easily be visualized on a TLC plate, at this and later stages of the synthesis, by using a Pasteur pipette to puff vapor from concentrated HCl over the plate: trityl-containing compounds turn orange.

▲ **CRITICAL STEP** Because of the acid sensitivity of the trityl-containing compound, it is advisable, at this and the next stage of the synthesis, to wash the silica gel with 0.5% (v/v) triethylamine in the elution solvent before running the flash chromatography column. Similarly, a small amount of triethylamine (1 μl) should be added to the CDCl₃ NMR solvent before dissolving the sample for analysis.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine 3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite ● **TIMING 12 h**

12| Place a three-neck round-bottom flask under a positive pressure of nitrogen, flame-dry it, and then allow it to cool down to room temperature under nitrogen.

13| Dissolve 520 mg (0.6 mmol) 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine in dry distilled THF (3 ml) under nitrogen in the flame-dried flask.

14| Add 0.71 ml (4 mmol) of diisopropylethylamine and 0.29 ml (1.3 mmol) of 2-cyanoethyl-N,N-diisopropylaminochlorophosphite (1.3 mmol) to the reaction using a syringe or by pipetman and allow the reaction to proceed for 6 h under nitrogen. Note that because the phosphite is very viscous, it is easiest to add to the reaction using a pipetman.

15| Pour the resulting mixture into 200 ml of ethyl acetate and wash the resulting solution three times with 5% (w/v) sodium bicarbonate (100 ml per wash) in a separatory funnel. Dry the organic layer over 10 g of anhydrous magnesium sulfate. Concentrate *in vacuo* using a rotary evaporator, and purify the residue by silica gel flash chromatography using 80% (v/v) ethyl acetate/hexanes as eluant ($R_f = 0.5$ – 0.6 , both diastereomers) to yield 622 mg (92%) of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite.

Synthesis of a caged hammerhead ribozyme substrate ● **TIMING 1 week**

16| Synthesize 5'GGGUGUA*UGGUU-3' (A* represents 2'-caged adenosine) using 2'-O-(2-nitrobenzyl) adenosine phosphoramidite and 2'-Fmp phosphoramidites on a 1 μmol scale using a DNA/RNA synthesizer. Use the standard RNA synthesis cycle with coupling times of 15 min for regular amidites and 30 min for the caged amidite.

▲ **CRITICAL STEP** Acid-labile Fmp phosphoramidites are used in this synthesis rather than silyl-modified *t*-butyldimethylsilyl (TBDMS) or bis(acetoxyethoxy)methyl (ACE) phosphoramidites (which are deprotected with fluoride) because of the fluoride sensitivity of the nitrobenzyl ether functionality. Test reactions showed that ~20% of the nitrobenzyl ether is removed upon treatment with fluoride under conditions used in deprotecting TBDMS-modified RNAs; thus, it is theoretically possible to use TBDMS chemistry in this protocol albeit with significantly lowered yields of caged RNA.

17| Cleave RNA from the resin and deprotect it by treatment with saturated ammonia/methanol (1 ml) in screw-cap tubes at 55 °C. Spin the reaction mixture to separate the resin from ammonia/methanol. Recover the supernatant and lyophilize to dryness using a speed-vac concentrator.

■ **PAUSE POINT** Incubate the lyophilized reaction mixture at 55 °C for 20 h.

18| Remove the 2'-Fmp groups by treatment with 500 μl NaOAc (pH 3.25).

28| Incubate for 3 h at 30 °C the 5'-phosphorylated synthetic branch oligomer (nucleotides 146–156; 300 pmol) with upstream PIP85.B RNA (nucleotides 1–145; 300 pmol) and 5' ³²P-labeled downstream (nucleotides 157–234; 100 pmol) T7 RNA transcription products in the presence of a bridging DNA “splint” complementary to the RNAs (cDNA(169–136): 5'-GAGAGAAGAAGCC AGTCAGCACC CTCGAGACGAG-3'; 100 pmol) and 40 U T4 DNA ligase (60 mM Tris, pH 7.8, 20 mM MgCl₂, 36 U ribonuclease inhibitor, 1.2 mM ATP, 2.4% (v/v) PVP-40, 5 mM DTT).

29| Purify ligations directly by 15% (w/v) denaturing PAGE (see Step 20). The products are visualized by autoradiography (**Fig. 5**).

30| Extract the RNA from the gel slice (see Step 20), dissolve in a small volume of double-distilled water and store at –78 °C (the sample is stable for at least 6 months under these conditions).

Uncaging 2'-nitrobenzyl RNA

31| Caged RNAs may be photolyzed using a variety of light sources including an excimer laser, as in option A, described as applied to the hammerhead ribozyme synthesized in Steps 16–23, or arc lamp, see option B, described as applied to the longer pre-mRNA synthesized in Steps 24–30. Because the deprotection times are similar, either option is appropriate for a generic caged RNA sequence. Note that the procedures for the analysis of the products from options A and B are specific to the RNA sequences used (hammerhead ribozyme and pre-mRNA). In particular, the analytical approach detailed in option B relies on the presence of a ³²P radiolabel in both the caged and photolyzed pre-mRNA.

(A) Laser photolysis of caged hammerhead ribozyme substrate ● TIMING 2 h

- (i) In a cutoff pyrex NMR tube, prepare a solution containing caged hammerhead RNA (5 nmol), 50 mM Tris, pH 7.5 and 10 mM MgCl₂ in a 100 μl volume.
- (ii) Photolyze the solution in the NMR tube with 100 pulses (308 nm; 300 mJ per pulse; full beam: 1–3 cm) from a Lambda Physik EMG 201 MSC excimer laser.
▲ **CRITICAL STEP** Photolysis of the reaction results in heating of the reaction solution. Just before photolysis, cool the reaction mixture by placing the NMR tube on ice. Pyrex reaction vessels shield UV light of wavelengths shorter than 300 nm, which can initiate unwanted crosslinking reactions.
- (iii) Extract the reaction mixtures with phenol/chloroform/isoamyl alcohol and then ethanol-precipitate the RNA (see Step 19).

Nucleoside composition analysis of caged and photolysis products ● TIMING 1 day

- (iv) Incubate a 5 nmol (~20 μg) sample of caged or photolyzed RNA in a 60 μl reaction volume containing 0.2 mM ZnCl₂, 16 mM MgCl₂, 250 mM Tris, pH 6.0, 0.2 U snake venom phosphodiesterase (Pharmacia) and 4 U calf-intestinal alkaline phosphatase (NEB) at 37 °C for 8 h. The sample containing caged RNA should be protected from exposure to ambient light using aluminum foil.
■ **PAUSE POINT** The reaction may be left to proceed overnight.
- (v) Following digestion, inject the sample onto a reverse-phase C-18 HPLC column (Waters) with a gradient elution from 0.05 M triethylammonium acetate to 1:1 0.1 M triethylammonium acetate/acetonitrile (16 min; 1 ml min⁻¹). Peaks corresponding to U, G, A and the modified nucleoside 2'-O-(2-nitrobenzyl)adenosine (there are no cytosines in the specific caged RNA substrate) are identified by coinjection of nucleoside standards with the chromatogram monitored at 254 nm.

(B) Arc lamp photolysis of caged pre-mRNA substrate ● TIMING 2 h

- (i) In a cutoff pyrex NMR tube, prepare a 10 μl solution containing 50–100 × 10³ c.p.m. of caged pre-mRNA in 60 mM KCl and 10 mM HEPES, pH 8.0.
- (ii) Cool the tube to 0 °C in an ice bath, remove and irradiate the sample at a distance of 1 cm with a 1,000 W Oriel Xenon arc lamp for 4 s.

TLC analysis of caged pre-mRNA and photolysis products ● TIMING 6 h

- (iii) The strategy for synthesis of the caged pre-mRNA (see Step 29; **Fig. 5**) results in a product with a single ³²P label two nucleotides 3' to the site of RNA modification with the caging group; thus, digestion of the RNA with RNase T1 and RNase A should yield A^{2'nb}pCp* (for caged RNA; A^{2'nb} corresponds to caged adenosine, p* corresponds to radiolabel) or ApCp* (for photolyzed RNA; p* corresponds to radiolabel). Digest caged or photolyzed pre-mRNA (50–100 × 10³ c.p.m.) with 2 U each of RNase T1 and RNase A (20 μl reaction volume, 10 mM Tris pH 7) for 2 h at 37 °C.
■ **PAUSE POINT** The digestion may be left overnight.
- (iv) Concentrate the reaction to dryness using a speed-vac concentrator. Resuspend the residue in 4 μl of water and load onto a cellulose PEI TLC plate (4 × 10 cm) and elute for 1 h in 79:19:1 saturated (NH₄)₂SO₄/1 M NH₄OAc/isopropanol. The air-dried TLC plate is exposed to a Molecular Dynamics phosphor screen and then scanned using a Molecular Dynamics Storm 860 Phosphorimager.

ANTICIPATED RESULTS

Phosphoramidite synthesis: typical yield

The outlined synthesis of the caged phosphoramidite will yield enough caged phosphoramidite for approximately 21 μmol couplings on a DNA/RNA synthesizer with a typical yield of ~200 nmol for a 10-nucleotide caged RNA.

Phosphoramidite synthesis: analytical data

2'-O-(2-nitrobenzyl)-N⁶-benzoyladenine

¹H NMR (200 MHz, CDCl₃): δ (p.p.m.) 8.72 (s, 1H, H₈), 8.12 (s, 1H, H₂), 8.04 (d, 2H, NO₂-ArH), 7.85–7.82 (m, 1H, NO₂-ArH), 7.61–7.28 (m, 6H, ArH), 6.00 (d, 1H, 14H), 5.09–4.91 (m 2H, methylene), 4.71 (s, 1H, 34H), 4.65 (d, 1H, 24H), 4.21 (s, 1H, 44H), 3.87 (dd, 2H, 54H).

The 2' position of the nitrobenzyl group was confirmed by NOED NMR data.

FAB-HRMS: (m/z) [M+H⁺] calculated for C₂₄H₂₂N₆O₇, 507.1628; observed, 507.1612.

5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-nitrobenzyl)-N⁶-benzoyl adenosine

¹H NMR (200 MHz, CDCl₃): δ(p.p.m.) 8.61 (s, 1H, H₈), 8.16 (s, 1H, H₂), 7.97 (d, 2H, NO₂-ArH), 7.83 (d, 1H, NO₂-ArH), 7.51–7.14 (m, 15H, ArH), 6.76 (m, 4H, DMT-ArH), 6.18 (d, 1H, 14H), 5.05 (dd, 2H, methylene), 4.27 (d, 1H, 44H), 3.71 (s, 6H, methoxy), 3.64–3.35 (m, 2H, 54H).

FAB-HRMS: (m/z) [M+H⁺] calculated for C₄₅H₄₀N₆O₉, 809.29347; observed, 809.2913.

5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-nitrobenzyl)-N⁶-benzoyladenine-3'-O-(2-cyanoethyl-N,N-diisopropylamino) phosphoramidite (caged phosphoramidite)

¹H NMR (200 MHz, CDCl₃): δ(p.p.m.) 9.55 (bs, 2H, NH_i, NH_{ii}), 8.61 (d, 2H, H_{8i}, H_{8ii}), 8.23 (d, 2H, NO₂-ArH_i, NO₂-ArH_{ii}), 7.94 (s, 2H, H_{2i}, H_{2ii}), 7.75 (d, 4H, NO₂-ArH_i, NO₂-ArH_{ii}), 7.64 (d, 2H, NO₂-ArH_i, NO₂-ArH_{ii}), 7.51–7.12 (m, 30H, ArH_i, ArH_{ii}), 6.80–6.74 (m, 8H, DMT-ArH_i, DMT-ArH_{ii}), 6.26 (m, 2H, 1'H_i, 1'H_{ii}), 5.26–4.90 [m, 4H, methylene_(i), methylene_(ii)], 4.69 (m, 2H, 3'H_i, 3'H_{ii}), 4.46 (m, 2H, 2'H_i, 2'H_{ii}), 4.39 (m, 2H, 4'H_i, 4'H_{ii}), 4.09–3.99 (m, 4H, 5'H_i, 5'H_{ii}), 3.78–3.31 [m, 8H, ethylene, cyanoethyl_(i), cyanoethyl_(ii)], 3.70 [d, 12H, methoxy_(i), methoxy_(ii)], 2.49–2.33 [m, 4H, methine, iPr_(i), iPr_(ii)], 1.29–0.91 [m, 24H, methyl, iPr_(i), iPr_(ii)].

³¹P NMR (300 MHz, CDCl₃): δ, 151.27 p.p.m., d, 150.94 p.p.m. (85% (v/v) H₃PO₄ in H₂O as external standard).

FAB-HRMS: (m/z) [M+H⁺] calculated for C₅₄H₅₇N₈O₁₀P, 1,009.4013; found, 1,009.4019.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at <http://www.natureprotocols.com>

Rights and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

1. Allerson, C.R. & Verdine, G.L. Synthesis and biochemical evaluation of RNA containing an intrahelical disulfide crosslink. *Chem. Biol.* **2**, 667–675 (1995).
2. Mayer, G. & Heckel, A. Biologically active molecules with a “light switch”. *Angew. Chem. Int. Ed.* **45**, 4900–4921 (2006).
3. Chaulk, S.G. & MacMillan, A.M. Caged RNA: photo-control of a ribozyme reaction. *Nucleic Acids Res.* **26**, 3173–3178 (1998).
4. Chaulk, S.G. & MacMillan, A.M. Separation of spliceosome assembly from catalysis using caged pre-mRNA substrates. *Angew. Chemie Int. Ed.* **40**, 2149–2152 (2001).
5. Chaulk, S.G., Kent, O.A. & MacMillan, A.M. Photo-control of RNA processing, in *Dynamic Studies in Biology: Phototriggers, Photoswitches, and Caged Biomolecules* (eds. Goeldner, M. & Givens, R.) 495–513 (Wiley-VCH Weinham, Germany, 2005).
6. Uhlenbeck, O.C. A small catalytic oligoribonucleotide. *Nature* **328**, 596–600 (1987).
7. Branch, A.D. & Robertson, H.D. Efficient trans cleavage and a common structural motif for the ribozymes of the human hepatitis delta agent. *Proc. Natl. Acad. Sci. USA* **88**, 10163–10167 (1991).
8. Buzayan, J.M., Gerlach, W.L. & Bruening, G. Nonenzymatic cleavage and ligation of RNAs complementary to a plant-virus satellite RNA. *Nature* **323**, 349–353 (1986).
9. Guo, H.C. & Collins, R.A. Efficient trans-cleavage of a stem-loop RNA substrate by a ribozyme derived from *Neurospora* VS RNA. *EMBO J* **14**, 368–376 (1995).
10. Peebles, C.L. *et al.* A self-splicing RNA excises an intron lariat. *Cell* **44**, 213–223 (1986).
11. Konarska, M.M. *et al.* Characterization of the branch site in lariat RNAs produced by splicing of messenger-RNA precursors. *Nature* **313**, 552–557 (1985).
12. Kröck, L. & Heckel, A. Photoinduced transcription by using temporarily mismatched caged oligonucleotides. *Angew. Chem. Int. Ed.* **44**, 471–473 (2005).
13. Heckel, A. & Mayer, G. Light regulation of aptamer activity: an anti-thrombin aptamer with caged thymidine nucleobases. *J. Am. Chem. Soc.* **127**, 822–823 (2005).

14. Mayer, G. *et al.* Light-induced formation of G-quadruplex DNA secondary structures. *ChemBioChem* **6**, 1966–1970 (2005).
15. Ting, R., Lerner, L. & Perrin, D.M. Triggering DNAzymes with light: a photoactive C8 thioether-linked adenosine. *J. Am. Chem. Soc.* **126**, 12720–12721 (2004).
16. Tang, X.J. *et al.* Photoregulation of DNA polymerase I (Klenow) with caged fluorescent oligodeoxynucleotides. *Bioorg. Med. Chem. Lett.* **15**, 5303–5306 (2004).
17. Wenter, P. *et al.* Kinetics of photoinduced RNA refolding by real-time NMR spectroscopy. *Angew. Chem. Int. Ed.* **44**, 2600–2603 (2005).
18. Höbartner, C. & Silverman, S.K. Modulation of RNA tertiary folding by incorporation of caged nucleotides. *Angew. Chem. Int. Ed.* **44**, 7305–7309 (2005).
19. Tang, X.J. & Dmochowski, I.J. Phototriggering of caged fluorescent oligodeoxynucleotides. *Org. Lett.* **7**, 279–282 (2005).
20. Monroe, W.T. *et al.* Targeting expression with light using caged DNA. *J. Biol. Chem.* **274**, 20895–20900 (1999).
21. Shah, S., Rangarajan, S. & Friedman, S.H. Light-activated RNA interference. *Angew. Chem. Int. Ed. Engl.* **44**, 1328–1332 (2005).
22. Ohtsuka, E., Tanaka, S. & Ikehara, M. Studies on transfer ribonucleic-acids and related compounds. 16. Synthesis of ribooligonucleotides using a photosensitive orthonitrobenzyl protection for 2'-hydroxyl group. *Chem. Pharm. Bull.* **25**, 949–959 (1977).
23. Bartholomew, D.G. & Broom, A.D. One-step chemical synthesis of ribonucleosides bearing a photolabile ether protecting group. *J. C. S. Chem. Commun.* **2**, 38 (1975).
24. Ohtsuka, E. *et al.* Studies on transfer-RNA and related compounds. 37. Synthesis and physical properties of 2'- or 3'-O-(ortho-nitrobenzyl) nucleosides—the use of orthonitrophenyldiazomethane as a synthetic reagent. *Chem. Pharm. Bull.* **29**, 318–324 (1981).
25. Moore, M.J. & Sharp, P.A. Site-specific modification of pre-mRNA: the 2'-hydroxyl groups at the splice sites. *Science* **256**, 992–997 (1992).
26. MacMillan, A.M. *et al.* Dynamic association of proteins with the pre-mRNA branch region. *Genes Dev.* **8**, 3008–3020 (1994).
27. Ohtsuka, E., Tanaka, S. & Ikehara, M. Studies on transfer ribonucleic acids and related compounds. (IX) Ribo-oligonucleotide synthesis using a photosensitive o-nitrobenzyl protection at the 2'-hydroxyl group. *Nucleic Acids Res.* **1**, 1351–1357 (1974).
28. Query, C.C., Moore, M.J. & Sharp, P.A. Branch nucleophile selection in pre-mRNA splicing: evidence for the bulged duplex model. *Genes Dev.* **8**, 587–597 (1994).

