# Debranchase-resistant labeling of RNA using the 10DM24 deoxyribozyme and fluorescent modified nucleotides

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# **Electronic Supplementary Information (ESI)**

All chemicals and solvents were purchased from commercial suppliers and used without further purification. Unmodified DNA oligonucleotides were purchased from IDT (Coralville, IA), Sigma-Aldrich (Munich, Germany) or Microsynth (Balgach, Switzerland). GTP was purchased from Promega (Madison, WI) and fluorescent EDA-GTP, 2'-O-propargyl guanosine, sulfo-Cy3-azide, sulfo-Cy5-azide, 6-FAM-azide and 5-hexynyl phosphoramidite were purchased from Jena Biosciences (Jena, Germany). *Sp*-GTP<sup>S</sup> (**1a**) and *Rp*-GTP<sup>S</sup> (**1b**) were prepared according to published procedures<sup>1</sup> and compared to commercial reference material purchased from BioLog (Bremen, Germany). RNAs were prepared by solid-phase synthesis using 5'-O-DMT-2'-O-TOM-protected 3'- $\beta$ -cyanoethyl phosphoramidites of *N*<sup>6</sup>-acetyladenosine, *N*<sup>4</sup>-acetylcytidine, *N*<sup>2</sup>-acetylguanosine, and uridine. 5-Ethylthio-1*H*-tetrazol (ETT) was from Azco BioTech (USA). A complete list of oligonucleotides used in this study is located in **Table S1**.

Anion exchange HPLC was performed on an ÄKTA micro (GE Healthcare) using a Dionex DNAPac PA200 (2 x 250 mm) column at 60°C. Eluent A: 25 mM Tris HCl, pH 8.0, 6 M urea; Eluent B: 25 mM Tris HCl, pH 8.0, 0.5 M NaClO<sub>4</sub>, 6 M urea. Reversed phase HPLC experiments were performed on a Shimadzu HPLC system with a diode array detector using a Nucleosil 100-5 C18 HD (4 x 250 mm) column at 40 °C. Eluent A:

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100 mM TEAA in H<sub>2</sub>O; Eluent B: 20 mM TEAA in H<sub>2</sub>O:MeCN 1:4. Denaturing polyacrylamide gels (0.5 or 0.7 mm thick, 20x30 or 20x40 cm, 10-20% acrylamide, 7 M urea, 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) were run in 1x TBE buffer at 35 W. Bromophenol blue and xylene cyanol were used as dye markers. On preparative gels, oligonucleotides were visualized by UV shadowing on a TLC plate. Fluorescence gel images were recorded with a Biorad ChemiDoc MP with epi illumination using blue, green, and red LEDs; emission filters 530/28 (blue), 605/50 (green), and 695/55 (red), or on a Typhoon scanner (GE healthcare), which was also used for phosphor imaging.

#### Preparation of Fluorophore-Labeled UBC4 pre-mRNA

A [<sup>32</sup>P]-labeled, 209 nucleotide (nt) *UBC4* pre-mRNA was prepared by *in vitro* transcription using T7 RNA polymerase from a DNA template and in the presence of trace amounts of  $\alpha$ -[<sup>32</sup>P]-UTP following standard protocols.<sup>2</sup> The transcript was purified by denaturing polyacrylamide gel electrophoresis (PAGE), extracted from the gel, ethanol precipitated, and quantified by scintillation counting before deoxyribozyme labeling. The sequence of UBC4 is shown below with the site of 10DM24 labeling underlined.

The UBC4 pre-mRNA, 10DM24 deoxyribozyme (D1, Table S1), and R cofactor

(R $\Delta$ , **Table S1**) were annealed at a ratio of 1:10:30 in 5 mM HEPES-NaOH pH 7.5, 15 mM NaCl, 0.1 mM EDTA by heating to 95°C for 3 min and cooling to ~22°C for 15 min. Reaction buffer including Mg<sup>2+</sup> and Tb<sup>3+</sup> metallocofactors and Cy3-GTP (Jena Bioscience, Germany) was then added as previously described<sup>3</sup> except that 80 mM Mg<sup>2+</sup> was used and labeling was carried out at 22°C. Labeling was stopped by addition of a denaturing stop solution (95% deionized formamide, 50 mM EDTA, 0.025% dextran blue) and the labeled RNA was then gel-purified.

# Purification of S. cerevisiae Dbr1

Dbr1 was expressed and purified essentially as previously described.<sup>4</sup> Briefly, BL21 (DE3) competent cells were transformed with pET16b-Dbr1 (gift of B. Schwer) and grown overnight at 37°C with antibiotic selection (100  $\mu$ g/mL ampicillin). The following day a single colony was picked and grown in 10 mL lysogeny broth (LB)/ampicillin for 4 h at 37°C with shaking (220 rpm) prior to inoculation of 1L LB/ampicillin. The culture was grown until reaching OD<sub>600</sub> = 0.6-0.8, cooled at 4°C on ice for 30 min, and then absolute ethanol was added to a final concentration of 2% (v/v). Cells were then induced with 0.4mM IPTG overnight at 16°C, and the cell pellet was stored at -80°C until use.

The cell pellet was then resuspended in 50 mM Tris pH 7.4, 250 mM NaCl, 10% (w/v) sucrose, and 0.2 mg/ml lysozyme and mixed for 30 min at room temperature. The lysate was adjusted to 0.1% (v/v) Triton X-100 and sonicated three times to reduce viscosity. Insoluble material was removed by centrifugation and the resulting soluble protein was purified using a 1mL HisTrap HP column (GE Healthcare) that had been equilibrated with lysis buffer lacking lysozyme and containing glycerol instead of sucrose.

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Bound protein was washed with 50 mL buffer containing 25 mM imidazole and eluted using a linear gradient of 25-500 mM imidazole. Fractions containing Dbr1 were pooled, diluted 10-fold using low salt buffer (150mM NaCl) and subsequently purified using a 1 mL Heparin FF (GE Healthcare) column. Protein was eluted using a linear gradient of 150-750 mM NaCl and stored at -80°C until use.

#### Dbr1 Cleavage Assays

Branched RNA substrates were 5' radiolabeled using T4 polynucleotide kinase (PNK, New England Biolabs) and  $\gamma$ -[<sup>32</sup>P]-ATP for use in debranching experiments. Debranching reactions (20 µL) were performed in 50 mM Tris-HCl (pH 7.0), 4 mM MnCl<sub>2</sub>, 2.5 mM DTT, 25 mM NaCl, 0.01% (v/v) Triton X-100, 0.1 mM EDTA, 0.15% (v/v) glycerol, 200 fmol RNA substrate and initiated with 10 ng Dbr1 before incubation for 1 h at 23°C.<sup>4</sup> Aliquots were removed at specified time points, quenched by addition of stop buffer (95% deionized formamide, 10 mM EDTA), and frozen immediately in liquid nitrogen until analysis. RNAs were resolved by 20% acrylamide (19:1) denaturing PAGE and the gels were subsequently dried and exposed overnight to a phosphorimager screen. The screen was scanned using a Typhoon Imager (GE Healthcare) and band intensities were quantified using ImageJ software.<sup>5</sup> Fluorescent RNAs were visualized by scanning the gel with the Typhoon Imager prior to drying.

#### Synthetic procedures

# Bis(tri-n-butylammonium) pyrophosphate

Ion exchange resin Amberlite (H<sup>+</sup>) was filled into a plastic column ( $\emptyset$ 1 cm) up to a height of 5 cm. The resin was washed with methanol until the eluate was colourless. Afterwards, the resins were washed with nanofiltered water until a pH of 5.0 was reached. Tetrasodium pyrophosphate  $\cdot$  10 H<sub>2</sub>O (0.89 g, 2.0 mmol, 1.0 eq.) was dissolved in 10 mL nanofiltered water, loaded in the column, and incubated at ambient temperature for about 10 min before being slowly eluted into an ice-cold, stirred solution of tri-*N*-butylamine (TBA; 0.74 g, 0.95 mL, 4.0 mmol, 2.0 eq) in 4 mL ethanol. The pyrophosphate was eluted with nanofiltered water until a pH of 5.0 was reached. The clear liquid was then concentrated in vacuo (water bath T≤30 °C) and co-evaporated twice with 8 mL ethanol, thrice with 8 mL DMF and dried under high vacuum overnight. The product was obtained as a colorless, viscous liquid (0.86 g, 1.6 mmol, 79%).





Scheme S1. Synthesis of 2'-O-propargyl-modified  $\alpha$ -thiotriphosphate diastereomers (2) and fluorescently labeled analogs (3)

2'-O-propargylguanosine (10 mg, 31 µmol, 1.0 eq.) was co-evaporated thrice with 0.5 mL pyridine, dissolved in 1 mL of dry trimethyl phosphate (TMP) and transferred into a 10 mL two-neck round-bottom flask equipped with a magnetic stirring bar. Proton sponge (PS, 10 mg, 47 µmol, 1.5 eq.) was added and the solvent removed under high vacuum overnight. Further reaction steps were performed under an inert nitrogen atmosphere. Dried 2'-O-propargylguanosine and PS were dissolved in 0.5 mL of dry TMP (gently heated and sonicated) and the solution was cooled to 0 °C with an ice-water bath. A

solution of pre-dried PSCI<sub>3</sub> (9.0 mg, 5.4 µL, 53 µmol, 1.7 eq.) in dry TMP (0.1 mL) was slowly added and the mixture was stirred at 0 °C for 4.5 h. Afterwards, a mixture of dry bis(tri-n-butylammonium) pyrophosphate (0.4 M in dry DMF, 0.39 mL, 155 µmol, 5.0 eq.) and tri-n-butylamine (28.7 mg, 36.9 µL, 155 µmol, 5.0 eq.) was added dropwise to the reaction mixture and the solution was stirred at 0 °C for another 1.5 h. The reaction was quenched by the addition of 2 mL triethylammonium bicarbonate (TEAB) buffer (0.1 M, pH 7.5) and slowly warmed to room temperature (40 min). The aqueous phase was extracted thrice with 6 mL of EtOAc, concentrated in vacuo (water bath T≤30 °C) and purified by anion exchange chromatography using a linear gradient from 0.1 M to 1 M TEAB buffer in 10 CV on a DEAE Sephadex A25 column (1 x 12 cm). After evaporation, the Sp- and the Rp-diastereomers were separated via reversed phase HPLC on a Nucleosil 100-5 C18 HD column, 4 x 250 mm, 6% MeCN and 100 mM TEAA buffer (pH 6.8) at 40 °C. Yield (Sp-diastereomer 2a): 1.6 µmol (5%); yield (Rp-diastereomer 2b): 2.0 µmol (7%). HR-ESI-MS (neg) for each diastereomer: calculated for C<sub>13</sub>H<sub>17</sub>N<sub>5</sub>O<sub>13</sub>P<sub>3</sub>S<sup>-</sup>: 575.9762, found for 2a: 575.9755, for 2b 575.9757. An aliquot of each diastereomer was used for Cu(I)-catalzyed azide alkyne cycloaddition (CuAAC) to prepare fluorescently labeled analogs.

To a solution of 500 nmol  $\alpha$ -S-2'-*O*-propargyl-GTP (**2a** or 2**b**) (33.4 µL of 14.7 mM in H<sub>2</sub>O; 1.0 eq) in 50 µL H<sub>2</sub>O and 25 µL DMSO/*t*BuOH (3:1) were added 10.0 µL sulfo-Cy5-azide (100 mM in H<sub>2</sub>O; 2.0 eq). 5 µL of a freshly prepared solution of CuBr (100 mM in DMSO/<sup>t</sup>BuOH 3:1; 1.0 eq) was mixed with 10.0 µL TBTA (100 mM in DMSO/tBuOH 3:1; 2.0 eq), and added to the solution, to give a final volume of 100 µL (in total 40% of organic solvent). The mixture was incubated at room temperature for 4h and precipitated

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by addition of 12 times the volume of ice cold 2% NaClO<sub>4</sub> in acetone (centrifuged at 4 °C, 13200 rpm for 30 min). The crude sample was dissolved in water and purified by reversed phase HPLC on a Nucleosil 100-5 C18 HD column, 4 x 250 mm, 0 – 49% B in 9 CV at 40 °C (buffer A: 100 mM TEAA in H<sub>2</sub>O, buffer B: 20 mM TEAA in H<sub>2</sub>O:MeCN 1:4). The isolated product was lyophilized and redissolved in 100  $\mu$ L of water, and the concentration was determined by UV absorbance. Yield: *Sp*-Cy5-GTP<sup>S</sup> (**3a**): 290 nmol (58%), *Rp*-Cy5-GTP<sup>S</sup> (**3b**): 395 nmol (79%). ESI-MS (pos) for both diastereomers: calculated for C<sub>50</sub>H<sub>66</sub>N<sub>14</sub>O<sub>23</sub>P<sub>3</sub>S<sub>4</sub>: 1410.3, found for **3a**: 1410.7, for **3b**: 1410.7.

#### Solid-phase synthesis of RNA samples

As described previously,<sup>6</sup> all RNA sequences were prepared by solid-phase synthesis on custom primer supports at 0.7 µmol scale in conventional 3' $\rightarrow$  5' direction using standard 5'-DMT-2'-TOM or 2'-TBDMS protected 3'- $\beta$ -cyanoethyl RNA phosphoramidites. Deprotection was performed under standard conditions in two steps using methyl amine in H<sub>2</sub>O/EtOH, and TBAF in THF. All oligonucleotides were purified by PAGE and analyzed by anion exchange HPLC on a Dionex DNAPac PA200 column and molecular weight was confirmed by ESI-Ms.

# Fluorescein-labeling of 5'-alkynylated RNA by CuAAC

5'-hexynylRNA (3.0 nmol) and 5.0 equivalents of respective alkyne (15 nmol) were mixed in 5.0  $\mu$ L H<sub>2</sub>O. 1.0  $\mu$ L of DMSO/*t*BuOH 3:1 and 1.5  $\mu$ L of a freshly prepared solution of CuBr (20 mM in DMSO/*t*BuOH 3:1) were mixed with 3.0  $\mu$ L TBTA (20 mM in DMSO/*t*BuOH 3:1), and added to the aqueous solution, to give a final volume of 10  $\mu$ L (final RNA concentration: 300  $\mu$ M). The mixture was incubated at 37 °C for 4-6h in the dark. Nucleic acids were precipitated by adding 10% (v/v) of 3 M NaCl solution and 3 times the volume of ice-cold 100% ethanol. The sample was kept on dry ice for 15 min. After centrifugation at 4 °C, 13200 rpm for 30 min, the supernatant was removed and the pellet dissolved in 8  $\mu$ L water. The labeled RNA was purified by denaturing PAGE (20% polyacrylamide, 0.4 mm, 35 W, 2 h 15 min). The yields of the isolated RNA ranged from 10-30%.

#### DNA-catalyzed labeling reactions

#### Kinetic assay

The 5'-fluorescein-labeled target RNA (100 pmol), the corresponding deoxyribozyme and R<sub>Δ</sub> (sequences see **Table S1**) were mixed in a 1:1.1:1.5 ratio and annealed in 25 mM HEPES pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating to 95 °C for 2 min and slow cooling to room temperature over 15 min. The labeling reactions were performed in 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM KCl, 50  $\mu$ M NTP, 100  $\mu$ M Tb<sup>3+</sup>, 80 mM Mg<sup>2+</sup>, and 0-10 mM Cd<sup>2+</sup> (final concentration target RNA: 10  $\mu$ M). The reaction mixtures were incubated at 37 °C and aliquots of 1.0  $\mu$ L were removed at desired time points and quenched with stop solution (80% formamide, 1x TBE, 50 mM EDTA, 0.025% each bromophenol blue and xylene cyanol). The reaction process was analysed by PAGE (20% polyacrylamide, 1x TBE, 7 M urea, 0.4 mm, 35 W, 20x30 cm, 1h 45min). Gel images were recorded with a Biorad ChemiDoc MP and band intensities were analysed by ImageQuant. The yield versus time data were fit using an exponential fit (Y = Y<sub>max</sub>(1-e<sup>-kobs+t</sup>) or employing a linear fit (Y =  $k_{obs}$ ·t) for yields <10% at the final timepoint.

### Preparative assay

The unlabeled target RNA (2-4 nmol), the corresponding deoxyribozyme and R<sub>Δ</sub>short were mixed in a 1:1.1:1.5 ratio and annealed in 25 mM HEPES pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating to 95 °C for 2 min and slow cooling to room temperature over 15 min. Five equivalents of  $\alpha$ -S-GTP (or corresponding analog) with respect to target RNA was added and the reaction was performed at 37 °C for 3-5 h in 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM KCl, 100  $\mu$ M TbCl<sub>3</sub>, 80 mM MgCl<sub>2</sub>, and 1 mM CdCl<sub>2</sub>. The final concentration of target RNA was 10, 15 or 20  $\mu$ M, and the final concentration of  $\alpha$ -S-GTP (or analog) was therefore 50, 75 or 100  $\mu$ M. Nucleic acids were precipitated by adding 10% (v/v) of 3 M NaCl solution and 3 times the volume of ice-cold 100% ethanol. The sample was kept on dry ice for 15 min. After centrifugation at 4 °C, 13 200 rpm for 30 min, the sample was washed with 75  $\mu$ L 70% ethanol (centrifugation at 4 °C, 13200 rpm for 5 min), dried and the pellet dissolved in 8  $\mu$ L water. The labeled RNA was purified by denaturing PAGE (20% polyacrylamide, 0.4 or 0.7 mm, 20x30 cm, 35W). Isolated yields ranged from 45-66%. Aliquots of the isolated RNAs were 5<sup>r-32</sup>P-labeled using PNK and  $\gamma$ -<sup>32</sup>P-ATP and used for debranching assays as described above.

Table S1							
Oligo Name	Sequence	Notes					
D1	CCGTCGCCATCTCCCGTAGGTGAAGGGC	10DM24 variant used for labeling UBC4					
	<u>AG1'I'C</u> GGT'I'CCA <i>1'I'C</i> C <u>T'I'AGCAA'I'ACG</u>	(Fig. 2A, B)					
D2	<i>CCGTCGCCATCTC</i> CCGTAGGTGAAGGGC	10DM24 variant used for labeling R1-R4					
	GTGACGGTTCCATTCCCGTATTATCC <sup>1</sup>	(Fig. 2C, D, 4B)					
D3	<i>CCGTCGCCATCTC</i> CCGTAGGTGAAGGGC	10DM24 variant used for labeling R5					
	GTGAGGGTTCCA <i>TTC</i> CCGTATTATCC <sup>1</sup>	(Fig. 3, 4A)					
RΔ	GA AGA GAU GGC GAC GG	RNA cofactor for all kinetic 10DM24 reactions					
R∆short	GA AGA GAU GGC GAC	RNA cofactor for preparative 10DM24 reactions					
R1_BsA	$CCA$ HAA HAC CAC HCA $C^2$	RNA substrate for 10DM24 and Dbr1 (Fig. 2C, D,					
	GGA UAA UAC GAG UCA C	4B)					
R2_BsG	GGA UAA UAC G <u>G</u> G UCA C <sup>2</sup>	RNA substrate for 10DM24 and Dbr1 (Fig. 2C, D)					
R3_BsC	GGA UAA UAC GCG UCA C <sup>2</sup>	RNA substrate for 10DM24 and Dbr1 (Fig. 2C, D)					
R4_BsU	GGA UAA UAC GUG UCA C <sup>2</sup>	RNA substrate for 10DM24 and Dbr1 (Fig. 2C, D)					
R5_BsA	GGA UAA UAC GAC UCA C <sup>2</sup>	RNA substrate for 10DM24 and Dbr1 (Fig. 3, 4A)					

<sup>1</sup> binding sites upstream and downstream of branch-site target underlined, binding site for R $\Delta$  italics;

<sup>2</sup> underlined nucleotides represent branch sites for 10DM24 labeling

Name	sequence	mol. wt. calc.	Mol.wt found
RΔ	GA AGA GAU GGC GAC GG	5262.3	5261.4
R∆short	GA AGA GAU GGC GAC	4571.9	4572.0
R1_BsA	GGA UAA UAC GAG UCA $C^2$	5128.2	5127.1
R2_BsG	GGA UAA UAC G $\underline{G}$ G UCA C <sup>2</sup>	5144.2	5144.9
R3_BsC	GGA UAA UAC GCG UCA C <sup>2</sup>	5104.1	5105.0
R4_BsU	GGA UAA UAC GUG UCA C <sup>2</sup>	5105.1	5106.7
R5_BsA	GGA UAA UAC GAC UCA C <sup>2</sup>	5088.1	5088.6

 Table S1.continued. ESI-MS data of synthetic RNA oligonucleotides



**Figure S1**. 10DM24 branching reactions with different branch-site substrates R1-R4 using DNA D2, R $\Delta$  and unmodified GTP in the presence of 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM KCl, 80 mM Mg<sup>2+</sup>, 100  $\mu$ M Tb<sup>3+</sup>, at 37 °C.



**Figure S2**. HPLC analysis of purified  $\alpha$ -S-GTP and  $\alpha$ -S-2'-(*O*-propargyl)-GTP. A) Superimposed HPLC spectra of purified *Sp*- and *Rp*-diastereomers of  $\alpha$ -S-GTP. B) HPLC spectra of purified *Sp*- and *Rp*-diastereomer of  $\alpha$ -S-2'-*O*-propargyl-GTP.



**Figure S3.** Optimization of reaction conditions for 10DM24-catalyzed labeling of R5. PAGE analysis of GTP,  $\alpha$ -S-Sp- and  $\alpha$ -S-Rp-GTP attachment to RNA R5 in the presence of different Cd<sup>2+</sup>-concentrations. Conditions: R5: D3: R<sub>\(\Delta\)</sub> (1:1.1:1.5), 50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM KCl, 80 mM Mg<sup>2+</sup>, 100  $\mu$ M Tb<sup>3+</sup>, with or without Cd<sup>2+</sup>, 37 °C. *k*<sub>obs</sub> values and yields are given in Table S2.

	GTP (P <sup>o</sup> )		α-S-Sp-GTP		thio effect	rescue effect	α-S-Rp-G	α-S-Rp-GTP		rescue effect
[Cd <sup>2+</sup> ]	<i>k</i> obs <sup>a</sup>	yield <sup>b</sup>	Kobs	yield <sup>c</sup>	k <sub>0</sub> /k <sub>s</sub>	(ko/ks)⁻ /(k <sub>0</sub> /k <sub>S</sub> )⁺	kobs	yield <sup>c</sup>	k <sub>0</sub> /k <sub>S</sub>	(ko/ks) <sup>-</sup> /(k <sub>0</sub> /k <sub>S</sub> )+
-	~3	91%	0.008	57%	~375		0.0003*	9%	~10000	
1 mM	~3	92%	0.057	87%	~50	~8	0.008	56%	~375	~25
5 mM	~3	91%	0.076	88%	~40	~10	0.015	76%	~200	~50
10 mM	~2	86%	0.040	85%	~50	~8	0.007	57%	~285	~35

Table S2. Examination of labeling efficiencies for GTP-attachment under different [Cd2+].

<sup>a</sup>  $k_{obs}$  in min<sup>-1</sup>.  $k_{obs}$  values marked with \* are from a linear fit. For all  $k_{obs}$  values given as ~2 or 3 min<sup>-1</sup> the conversion was >60% at the second time point (30 sec), therefore no accurate curve fit was obtained. <sup>b</sup>. final yield in % at 2h. <sup>c</sup>. final yield in % at 7h.



**Figure S4.** HPLC analysis of Cy5- and fluorescein-labeled R1 used in Fig 4B. Dionex DNAPac PA200 column, 2 x 250 mm, 0 - 48% B in 12 CV, 60 °C; eluent A: 25 mM Tris HCl, pH 8.0, 6 M urea; eluent B, 25 mM Tris HCl, pH 8.0, 0.5 M NaClO<sub>4</sub>, 6 M urea; detection at 260 nm (black), 496 nm (red), 647 nm (blue). Alk-G = 2'-(*O*-propargyl)-guanosine, FI = fluorescein. A) Chromatograms of reaction products with *Rp*-GTP<sup>s</sup> substrates, i.e. 2',5'-Sp-linked RNA-GMP products. B) Chromatograms of reactions with *Sp*-GTP<sup>s</sup> substrates.

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Appendix: ESI-MS spectra of compounds 2a,b and 3a,b



Figures S5. HR ESI-MS of 2'-O-propargyI-Sp-GTP<sup>S</sup> (2a)



Figures S6. HR ESI-MS of 2'-O-propargyl-*Rp*-GTP<sup>S</sup>(2b)



Figures S7. ESI-MS of 2'-Cy5-labeled-*Rp*-GTP<sup>S</sup>(3a)



Figures S8. ESI-MS of 2'- Cy5-labeled-*Rp*-GTP<sup>S</sup>(3b)