Supporting information

An RNA modification with remarkable resistance to RNase A

Alice Ghidini, Charlotte Ander, Anna Winqvist and Roger Strömberg*

Department of Biosciences and Nutrition, Unit for Bioorganic Chemistry,
Karolinska Institutet, S-14183 Huddinge, Sweden

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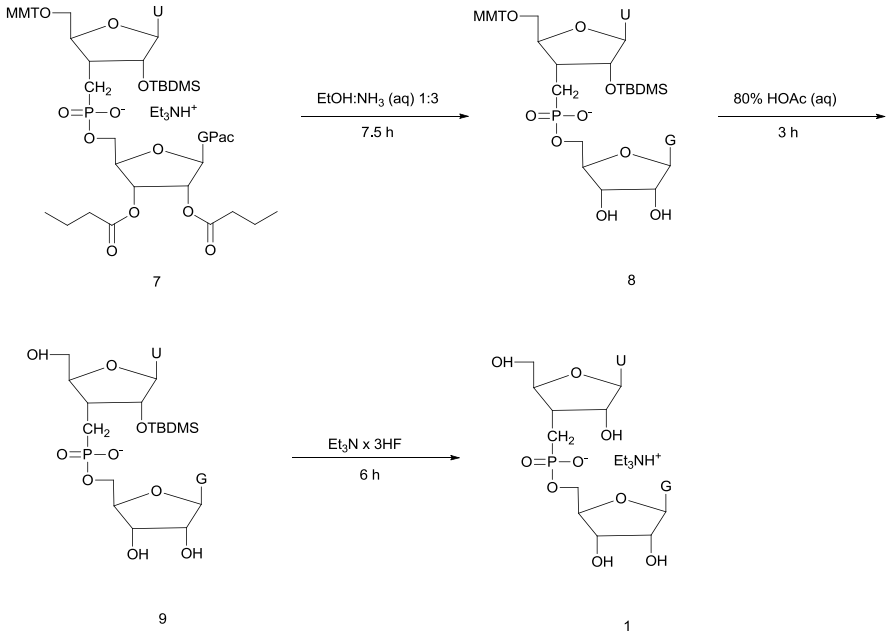
S1. MATERIALS AND METHODS

All buffers used for kinetics were prepared using deionized water and the pH of all aqueous solutions was measured (Orion 330 pH-meter) at room temperature.
The 3-morpholinopropane-1-sulfonic acid (MOPS), tris(hydroxymethyl)aminomethane chloride (Tris HCl), aqueous ammonia, triethylamine trihydrofluoride, PDE II from bovine spleen, and RNase A type IX-X were purchased from Sigma Aldrich, acetic acid, NaOH pellets, NaCl, $MgCl_2$ were purchased from Fluka. The UpG dinucleotide was purchased from IBA Nucleic Acid Synthesis and PDE I from Crotalus adamanteus venom was purchased from USB Corporation. The reference compounds uridine, guanosine, guanosine 5'-phosphate, uridine 3'-phosphate and uridine 2',3'-cyclic phosphate were purchased from Sigma. All solvents and reagents used were of analytical quality and purchased from commercial sources.

NMR spectra were recorded on a Bruker AVANCE DRX-400 instrument (400.13 MHz for ^{1}H, 162.00 MHz for ^{31}P) and proton chemical shifts are reported relative to the residual solvent peak (4.8 for HDO) or 1% phosphoric acid in D_2O (0.0 ppm). Reverse phase HPLC vas carried out on a Jasco HPLC system, equipped with autosampler, oven and UV detector Jasco. Mass spectrometry was performed on a Micromass LCT electrospray time-of-flight (ES-TOF) in acetonitrile:water=1:1 solutions. The dimer concentrations were measured at 260 nm on a Cary 300 UV-Vis dual beam spectrophotometer (Varian). Concentration of the UpG (**2**) and the modified dimer UCH_2pG (**1**) were determined by UV spectroscopy using for both of them ε = 20800 M^{-1} cm^{-1} at 260 nm (M.J. Cavaluzzi, P.N. Bore, *Nucleic Acid Research*, **2004**, 32,1-13; Cantor C.R., Warshaw M.M., Shapiro H., *Biopolymers*, 9,1059-1077).

S2. PREPARATION OF UCH_2pG (1)

Compound **7** was prepared as described (A. Winqvist, R. Strömberg, Eur. J. Org. Chem., 2008, 1705-1714) and then deprotected and purified as described below.



7 8 9 1

Scheme S1. Deprotection scheme for preparation of **1**.

Compound **7** (30 mg, 22.2 μmol) was suspended in a solution of 32% aqueous ammonia (3 ml) and 99.7% ethanol (1 ml), and the reaction mixture was stirred for 7.5 h at room temperature. The mixture was concentrated under reduced pressure (< 30 °C). The residue (crude compound **8**) was dissolved in 80% aqueous acetic acid (2 ml), and the reaction mixture was left for 3 h at room temperature. The mixture was diluted with water (5 ml) and was washed with diethyl ether (2×5 ml). The combined organic layer was washed with water (3 ml). The combined water layer was concentrated under reduced pressure (< 30 °C) to remove traces of organic solvents, and was then lyophilized. The residue (crude compound **9**) was suspended in neat triethylamine trihydrofluoride (0.5 ml). The reaction mixture was left for 6 h at room temperature. The mixture was diluted with water (5 ml) and was washed with ethyl acetate (2×5 ml). The combined organic layer was washed with water (3 ml). The combined water layer was concentrated under reduced pressure (< 30 °C) to remove traces of organic solvents, and was then lyophilized. Most of the remaining triethylamine trihydrofluoride were removed by lyophilizing a solution of the residue dissolved in water (10 ml), a procedure that was repeated to give a solid residue. The product (crude compound **1**) was dissolved in 0.05 M aqueous triethylammonium acetate, pH adjusted to 6.5 (400 μl), and was triturated by addition of acetonitrile (2.6 ml). The mixture was kept at 0 °C for 20 h, and was then centrifuged. The supernatant was removed and the solid residue was washed with acetonitrile (3×2.5 ml) and subsequently dissolved in water (2 ml). The solution was filtered trough a plug of glass wool and was lyophilized. The solid residue contained pure compound **1** according to ^{1}H- and ^{31}P-NMR analyses (no organic salts or other side products could be observed), however, the product contained 1.5 equivalents of the counter ion and was therefore lyophilized again from water (2 ml) to give 17 mg of white solid (**1**+salts). The product was additionally purified by reversed-phase HPLC equipped with a Jones AQ (5 μm, 150x4.6 mm) column and using a linear gradient of (A) 50 mM triethylammonium acetate in water (pH=6.5) and (B) 50 mM triethylammonium acetate in 50% aqueous acetonitrile (pH=6.5). The gradient was 0-20% buffer B over 20 min, a flow rate of 1 ml/min was used and the chromatography was performed at 50 °C with UV detection at 260 nm. Mass calculated for compound **1** [M^-], 586.1299; found, 586.1278; ^{1}H NMR (d_2O): δ 8.11 (bs, 1H, H_8-G); δ 7.91 (d, 1H, ^{3}J= 8.1, H_6-U); δ 5.88 (1H, d, ^{3}J = 4.3, 1´-G); δ 5.62 (s, 1H, 1´-U); δ 5.61 (d, 1H, ^{3}J = 8.2, H_5 U); δ 4.54 (t, 1H, ^{3}J = 4.6, 2´-G); δ 4.42 (m, 2H, 2´-U+3´-G); δ 4.27 (m, 1H, 4´-G); δ 4.16 (dt, 1H, ^{2}J = 11.8, ^{3}J = 3.1, 5´-G); δ 4.07 (dt, 1H, ^{2}J = 11.8, ^{3}J = 4.1, 5´´-G); δ 3.89-3.98 (m, 2H, 4´-U and 5´´-U); δ 3.73 (dd, 1H, ^{2}J =11.6, ^{3}J = 3.5, 5´-U); δ 3.16 (q, 6H, ^{3}J=7.3, CH_2-TEAH$^+$); δ 2.20 (m, 1H, 3´-U); δ 1.94 (m, 1H, CH_2-P); δ 1.56 (m, 1H, CH_2-P); δ 1.24 (t, 9H, ^{3}J=7.3, CH_3-TEAH$^+$).^{31}P NMR (d_2O): δ 25.0.

S3. ASSAYS FOR ENZYMATIC AND BASE CATALYZED CLEAVAGE OF DIMERS UCH$_2$pG (1) AND UpG (2)

A) Assay for cleavage of dinucleotides with PDE I.

The reactions were performed in 1.5 ml plastic tubes with screw cap immersed in a water bath at 37 °C. Incubation was carried out in a buffer with the following recipe: 110 mM Tris HCl, 1M HCl, 100 mM NaCl, 14 mM $MgCl_2$ at pH 8.9. The final concentration of the enzyme (snake venom phosphodiesterase I, SVPD, from *Crotalus Adamanteus*) used was 0,015 U/ml and it was maintained constant for all the experiments as was the concentration of the UpG (c=0.069 mM) and UCH$_2$pG

(c=0.069mM). The samples were prepared at room temperature adding first the enzyme stock solution to the tube followed by 891 µl of Tris buffer, the amount of water necessary to achieve the right concentration and last the aliquot from the UpG or UCH_2pG stock solutions. The reaction mixture was vortexed for 10 seconds and placed in the water bath. The final volume of the sample was 500 µl and 6 aliquots were collected at specific times (80 µl each one), quenched with 20 µl of 75 mM EDTA solution, centrifuged for 20 min with Microcon YM-3 filter tubes in order to remove the enzyme. The samples were frozen and stored at -18 °C until being analyzed by HPLC.

B) Assay for cleavage of dinucleotides with PDE II.

The reactions were performed in 1.5 ml plastic tubes with screw cap immersed in a water bath at 37 °C. The incubations were carried out in a buffer with the following recipe: 250 mM Na succinate, 1 M HCl at pH 6.5. The final concentration of the enzyme used was 0.03 U/ml and it was maintained constant for all the experiments as was the concentration of UpG (c=0.069 mM) and UCH_2pG (c=0.069 mM). The samples were prepared at room temperature adding first the enzyme stock solution to the tube followed by 940 µl of buffer, the amount of water necessary to achieve the right concentration and last the aliquot from the UpG or UCH_2pG stock solutions. The reaction mixture was vortexed for 10 seconds and placed in the water bath. The final volume of the sample was 1 ml and 7 aliquots were collected at specific times (100 µl each one) and centrifuged for 8 min with Ultrafree-MC 5000 filter tubes in order to remove the enzyme. The samples were frozen and stored at -18 °C until being analyzed by HPLC.

C) Assay for cleavage of dinucleotides with RNase A.

The reactions were performed in 1.5 ml plastic tubes with screw cap immersed in a water bath at 37 °C. Incubation was carried out in 53 mM MOPS buffer at pH 7. The final concentration of the enzyme used was 0,05 U/ml and it was maintained constant for all the experiments as was the concentration of UpG (c=0.069 mM) and UCH_2pG (c=0.069 mM). The samples were prepared at room temperature adding first the enzyme to the tube followed by the MOPS buffer, the amount of water necessary to achieve the right concentration and last aliquots from the UpG or UCH_2pG stock solutions. The reaction mixture was vortexed for 10 seconds and placed in the water bath. The final volume of the sample was 800 µl and 6-9 aliquots were collected at specific times (90 µl each one), centrifuged for 8 min with Ultrafree MC 5000 filter tubes in order to remove the RNase A and stop the reaction. The samples were frozen and stored at -18 °C until being analyzed by HPLC.

D) Competition experiments with RNase A.

The experiments were performed under the same conditions as described for the cleavage assays above, but with both UpG and UCH_2pG present in different proportions: the concentration of the UpG (c=0.069 mM) was maintained constant as was the concentration of the RNase A (c=0.05 U/ml), whereas the concentration of the modified dimer UCH_2pG was varied (0, 0.069, 0.345 and 0.69 mM) depending on the experiment. The final volume of the sample was 350 µl and 6 aliquots were collected at specific times (50 µl each one), diluted with 50 µl of water and centrifuged for 8 min with Spin-X UF 5k Corning filter tubes. The samples were frozen and stored at -18 °C until being analyzed by HPLC.

E) Assay for base catalyzed cleavage of dinucleotides.

The base catalyzed cleavage was studied at two different conditions 0.01M (with 0.19 M NaCl to make the ionic strength 0.2 M) and 0.05 M NaOH (with 0.15 M NaCl). The samples were prepared at room temperature adding first the NaOH solution to the tube whereupon the aliquots from the UpG or UCH_2pG stock solutions were added. The reaction mixture was vortexed for 10 seconds and placed in the water bath at 50 °C. The final volume of the sample was 200 µl and 9 aliquots were collected at specific times (15 µl each one) and diluted with 7.5 µl of water and 7.5 µl of HCl 0.1 M solution for the cleavage with buffer at pH= or diluted with 15 µl of HCl 0.01 M solution in order to reach neutral pH and stop the reaction. The samples were frozen and stored at -18 °C until being analyzed by HPLC.

F) HPLC analysis

The aliquots were analyzed by reversed-phase HPLC equipped with an ODS Hypersil C18 (5 µm, 3x250 mm) column and using a linear gradient of (A) 50 mM triethylammonium acetate in water (pH=6.5) in (B) 50mM triethylammonium acetate in 50% aqueous acetonitrile (pH=6.5). The analyses were performed using a gradient 0-20% buffer B over 20 min with a flow rate of 0.43 ml/min at 25 °C and UV detection at 260 nm. The fraction of remaining uncleaved dinucleotide, quantified by integration of the HPLC chromatograms, was plotted against time. For the base catalyzed reactions the first order constant k was calculated by linear fitting of the natural logarithm of the inverse of the remaining fraction of dimer.

S4. ADDITIONAL GRAPHS (WITH LONGER TIME SCALES) OF PERCENTAGE OF REMAINING DINUCLEOTIDES AT DIFFERENT TIMES UPON TREATMENT OF UCH_2pG (1) AND UpG (2) WITH SPLEEN EXONUCLEOASE (PDE II) AND RNASE A.

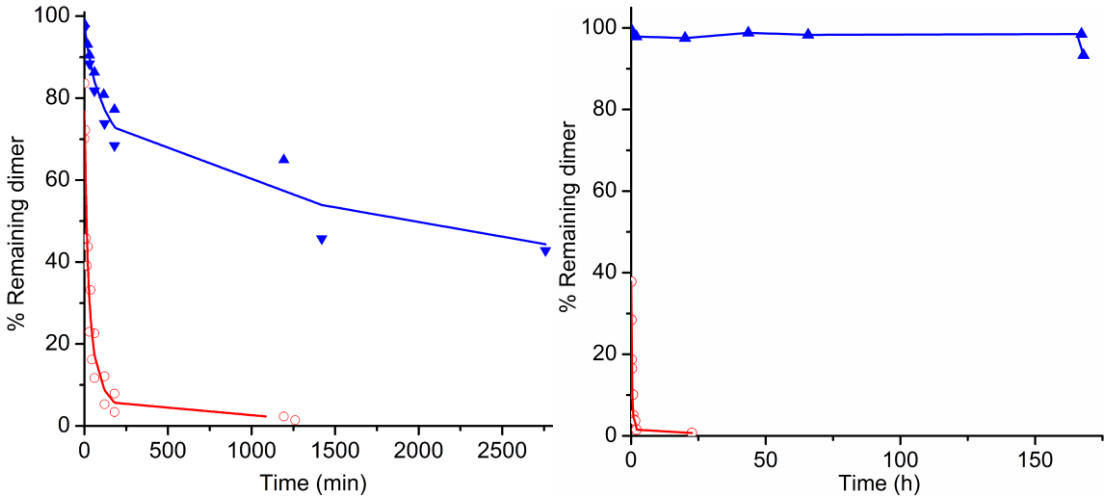


Figure S1. Graphs showing % remaining dinucleotide (UCH_2pG in blue and UpG in red) at different times, when subjected to Spleen exonuclease, PDE II (left panel) or RNase A (right panel) at 37 °C. Quantification of dinucleotide and product was done by integration of the RP-HPLC analysis of the mixtures.

S5. EXAMPLES OF HPLC ANALYSIS FROM THE DIFFERENT REACTIONS OF DINUCLEOTIDES UCH_2pG (1) AND UpG (2):

A) Example of chromatogram from HPLC analysis of the treatment of UpG (**2**) and UCH_2pG (**1**) (0.069 mM) with snake venom phosphodiesterase from Crotalus Adamanteus (SVPD, PDE I, 0.015 U/ml):

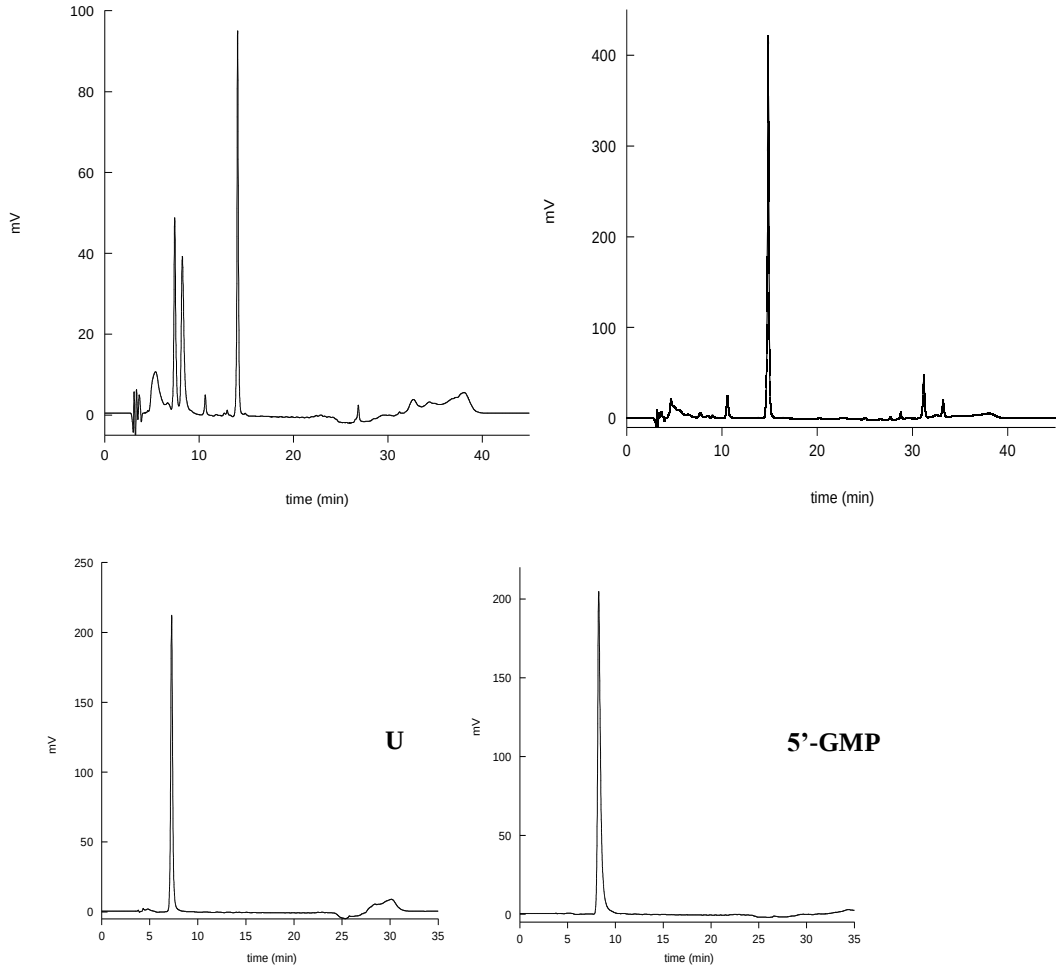


Figure S2: Upper left panel: Treatment of UpG with PDE I after 5 min of incubation; Upper right panel: Treatment of UCH_2pG with PDE I after 2 days incubation. The RP-chromatogram shows that there is no degradation of the modified dimer when incubated with the enzyme. Lower left panel: HPLC-analysis of uridine (U). Lower right panel: HPLC-analysis of guanosine 5'-phosphate (5'-GMP)

B) Examples of chromatogram from HPLC analysis of the treatment of UpG (**2**) or UCH_2pG (**1**) (0.069 mM) bovine spleen PDE II (c = 0.03 U/ml)

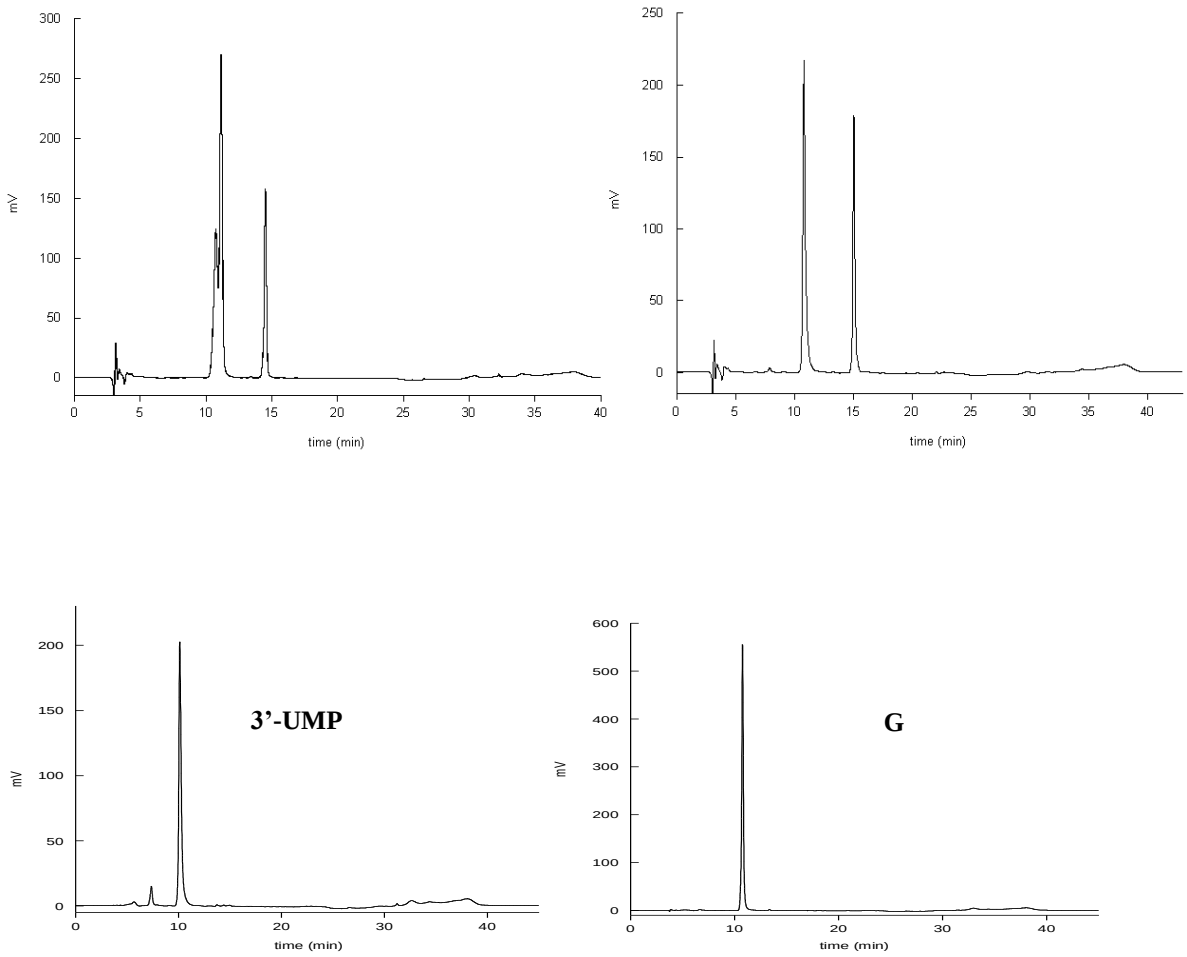


Figure S3: Upper left panel: Treatment of UpG with PDE II for 60 min; Upper right panel: Treatment of UCH_2pG with PDE II for 20 h. Lower left panel: HPLC-analysis of uridine 3'-phosphate (3'-UMP). Lower right panel: HPLC-analysis of guanosine (G).

C) Examples of chromatograms from HPLC analysis of the treatment of UpG (**2**) and UCH_2pG (**1**) (0.069 mM) with RNase A (c = 0.05 U/ml)

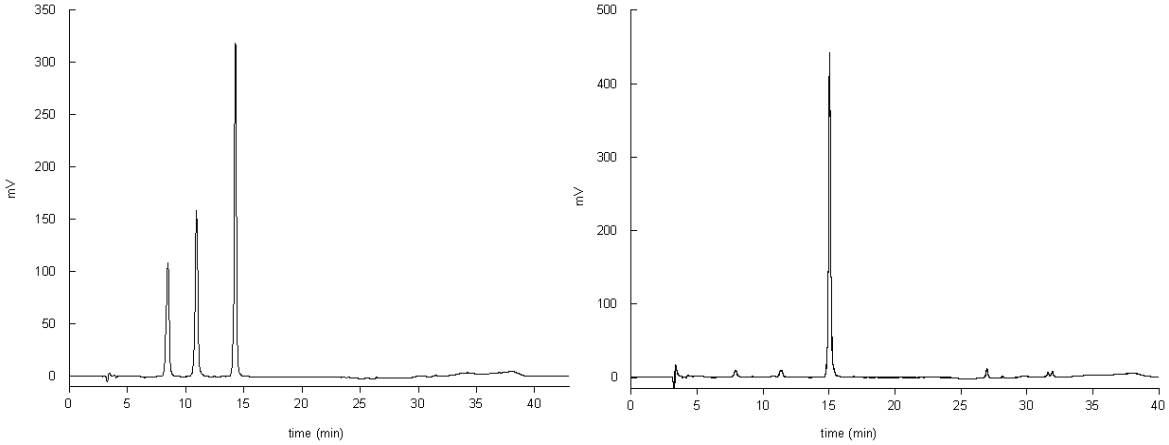


Figure S4: Left panel: Treatment of UpG with RNase A for 4 min; Right panel: Treatment of UCH_2pG with RNase A for 7 days.

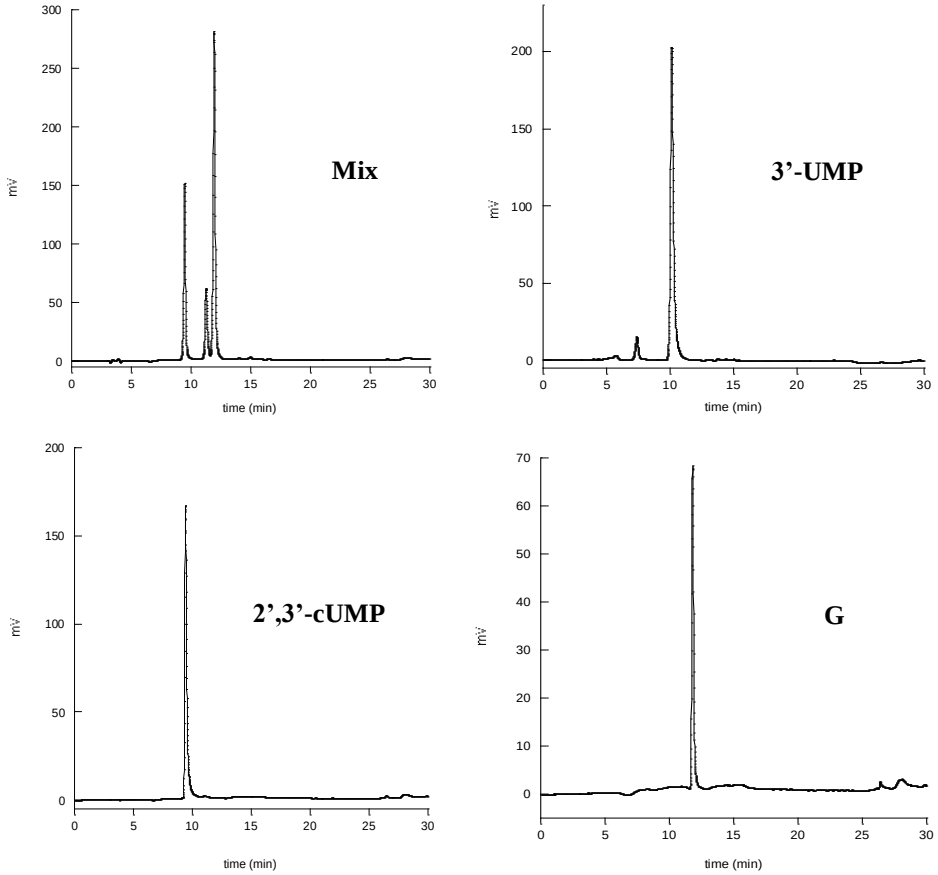
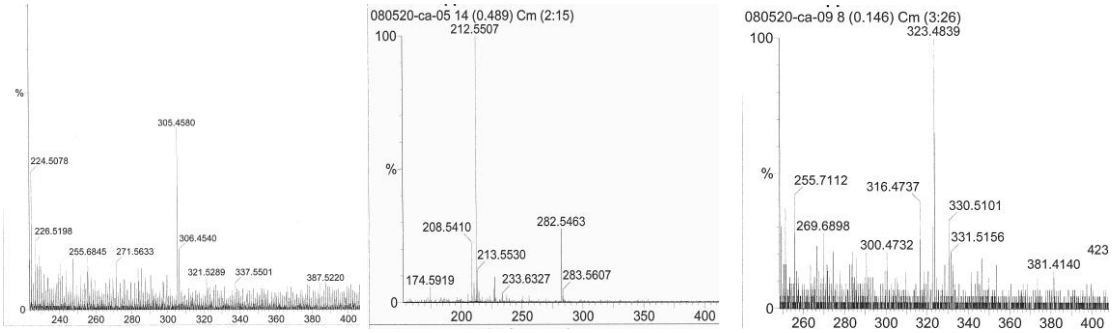


Figure S5: Verification of cleavage products after complete cleavage (Mix , 6-fold higher conc. (0.3 U/ml) of RNase A after 30 s) by comparison to commercial guanosine (G), uridine 3'-phosphate (3'-UMP) and uridine 2',3'- cyclic phosphate (2',3'-cUMP).



2',3'-cUMP **G** **3'-UMP**

Figure S6: Mass spectra of the three product peaks from the Mix in Figure S5 additionally verifying the identity of the cleavage products.

D) Example of chromatogram from HPLC analysis of the treatment of UCH_2pG (**1**) (0.069 mM) with ten times higher concentration of RNase A (c = 0.5 U/ml, i.e., 10 times higher concentration of enzyme than in the previous experiment).

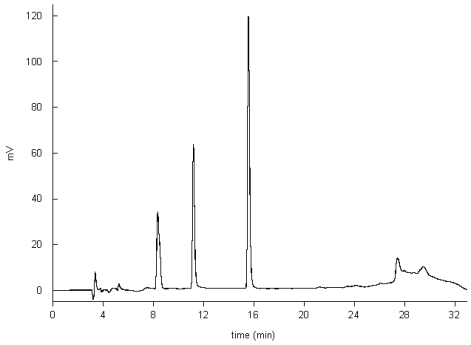


Figure S7: Treatment of UCH_2pG with 10xhigher concentration of RNase A (0.5 U/ml) for 60 min.

E) Example of chromatograms from HPLC analysis of the competition experiments with RNase A. RNase A (c = 0.05 U/ml) catalyzed cleavage of UpG (1) (0.069 mM) was performed in absence or presence of UCH_2pG(2) (0.345 mM, 5 equiv.).

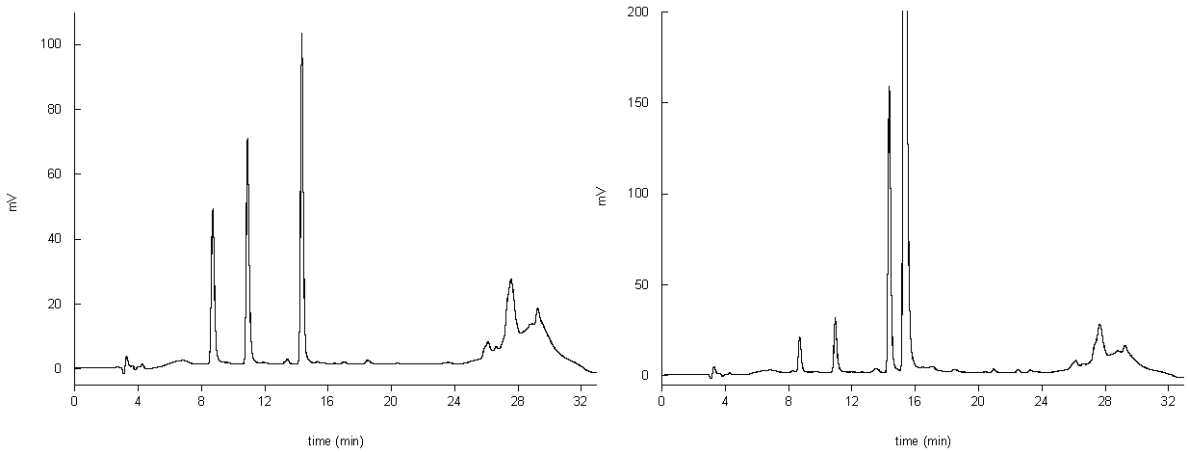


Figure S8: Left panel: Treatment of UpG with RNase A (0.05 U/ml) for 13 min; Right panel: Treatment of UpG in presence of UCH_2pG (in a ratio of 1:5) with RNase A (0.05 U/ml) after 13 min.

F) Example of chromatograms from HPLC analysis of base catalyzed cleavage (0.05 M NaOH, 0.15 M NaCl) of UpG(**1**) and UCH_2pG(**2**) (0.069 mM).

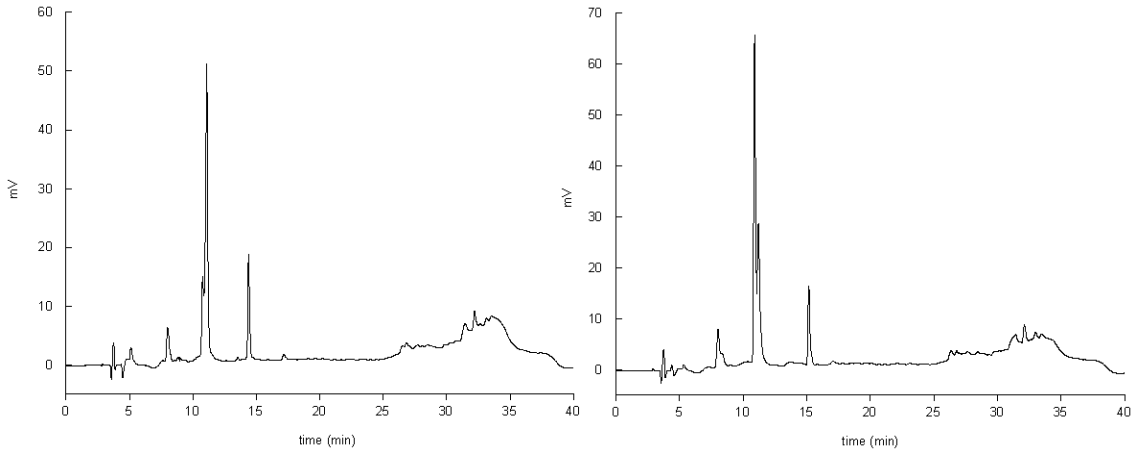


Figure S9: Left panel: Treatment of UpG for 2 h with 0.05 M NaOH; Right panel: Treatment of UCH_2pG for 4 h with 0.05 M NaOH.