

## Evaluation of Chemical Labeling Strategies for Monitoring HCV RNA using Vibrational Microscopy

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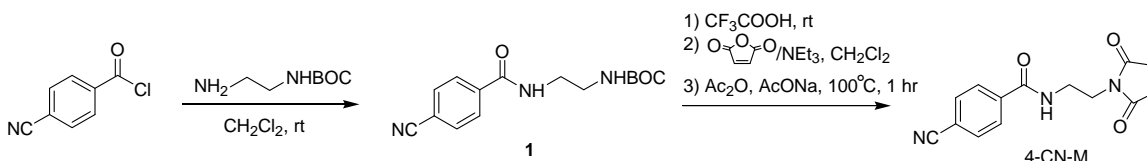
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## 1. HPLC/LC-MS Analysis of Normal and Modified HCV RNA

In order to separate and quantify the relative proportions of modified and unmodified RNA bases, HPLC conditions were first optimized to distinguish the individual monomeric nucleotides (Figure S8). After several trials we determined that the following conditions were optimal: 0.1 M NH<sub>4</sub>OAc, pH 6.5 at a flow-rate of 0.2 mL/min. Before the optimized HPLC/LC-MS conditions were applied to the analysis of modified RNAs, digestion and dephosphorylation of HCV RNA and aaU-HCV RNA were performed to obtain the constituent mononucleosides. HPLC/LC-MS analysis clearly showed that the mononucleoside components of HCV RNA and aaU-HCV RNA were resolved following digestion and dephosphorylation as described in the materials and methods (Figure S2).

To determine if the digestion conditions were achieving maximal sample decomposition, the concentration and time dependence of the reaction was examined. The results of the concentration dependence experiment showed that the HCV RNA and aaU-HCV RNA reactions appeared to saturate with 10 – 25U of S1 nuclease (Figure S9A). Furthermore, the time dependence experiment showed that the digestion reaction saturated after 1 hour (Figure S9B).

## 2. Synthesis of 4-CN-M

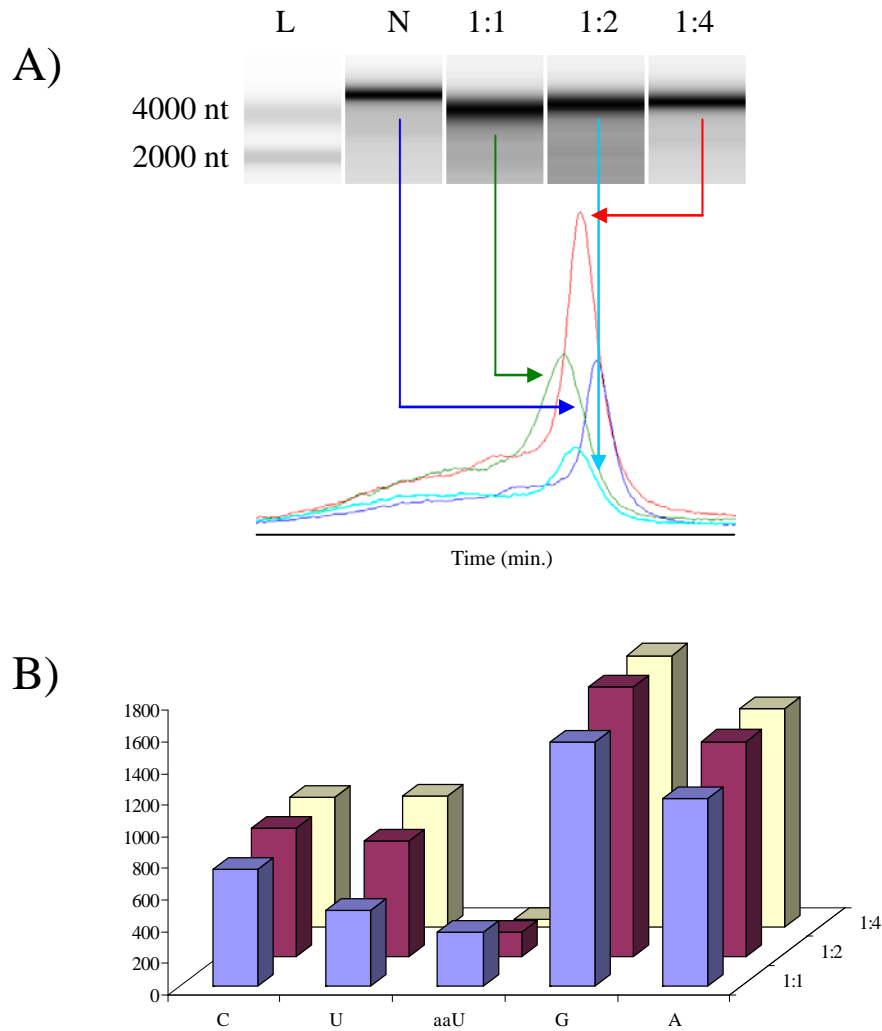


4-Cyano-benzoyl chloride (300 mg, 1.8 mmol) was suspended in 6 mL dichloromethane. N-BOC-1,2-ethylenediamine (320  $\mu$ L, 2.0 mmol) and 300  $\mu$ L triethylamine in 6 mL dichloromethane was added under argon. The mixture was stirred for 30 min and then rotavaped to dry. The residue was triturated in 5 mL ethyl acetate. The precipitate was collected by vacuum filtration to give crude **1**, N-BOC-1,2-(4-cyanobenzamido)ethylenediamine (432 mg, 83%). Crude **1** (289 mg, 1.0 mmol) was dissolved in 0.8 mL trifluoroacetic acid and stirred overnight. The volatile components were removed under vacuum. The residue was added 200  $\mu$ L triethylamine and dissolved in 2 mL dichloromethane under argon. Maleic anhydride (108 mg, 1.1 mmol) was added and the mixture was stirred for 45 min under argon and then rotavaped to dry. The residue was triturated in 2 mL water. The precipitate was collected by vacuum filtration and washed with 2 mL of ethyl acetate to give 135 mg of white solid (47%). This white solid was added 0.8 mL acetic anhydride and 45 mg of anhydrous sodium acetate. The mixture was heated at 100 °C for 1 hr. After cooling down, 15 mL ice water was added and the mixture was stirred for another hour. Then it was brought to basic by added sodium carbonate. The precipitate was collected by vacuum filtration and purified by silica column chromatography (8:2 of ethyl acetate: hexane) to give final product **3**. <sup>1</sup>H NMR (400 MHz) 7.97 (d, 2H), 7.89 (d, 2H), 6.87 (s, 2H), 3.74 (t, 3H), 3.64 (t, 2H). FAB-MS (M+1) calculated 270.1; found 270.1.

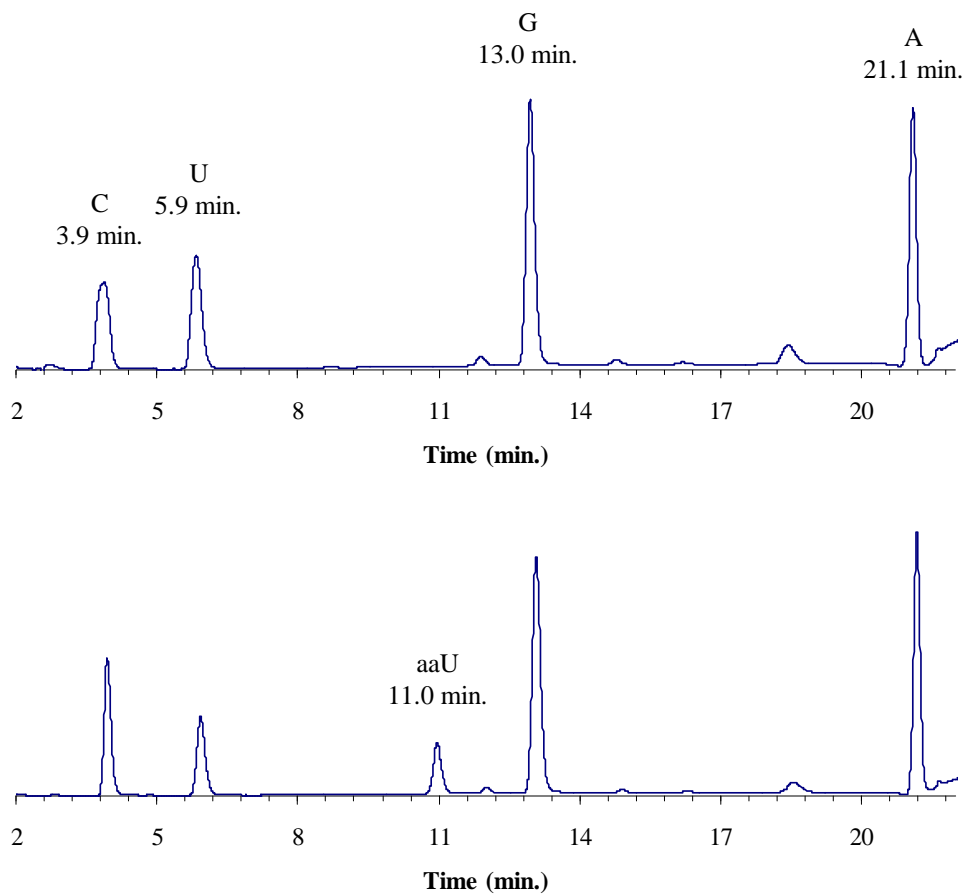
### 3. CN Modification of aaU-HCV RNA

To overcome stereoelectronic effects in the cyano-labeling of HCV RNA we utilized 3-cyanobenzyl-NHS to label aaU-163mer. Again, initial analysis of the electrophoretic migration showed an increase in the apparent mass of labelled aaU-163mer, which was consistent with a successful reaction (Figure S4A). HPLC comparison to control reactions clearly showed the appearance of a new product at 23.6 min., with a corresponding disappearance of the aaU peak at 10.5 min., both of which were indicative of a successful reaction (Figure S4C). ESI-MS analysis of the digested nucleoside mixture showed a signal for the 3-CN-modified aaU (aaU-CN;  $m/z = 429$ ).

Electrophoretic analysis of CN-HCV RNA revealed a mass-shift that was consistent with those seen for Bz-NHS and 3-CN-NHS modified aaU-163mer (Figure S5A). Furthermore, the presence of the peak believed to be aaU-CN was immediately apparent at 23.6 min (Figure S5B). We conclude that 3-CN-NHS successfully modified aaU-HCV RNA.

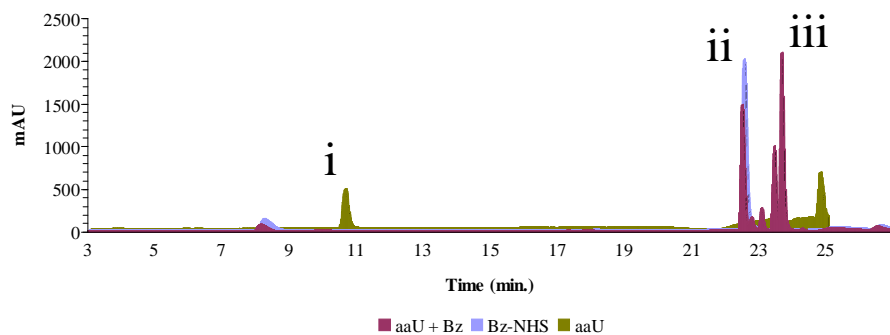


**Figure S1.** Regulating the extent of aaU incorporation. A) Electrophoretic migration of HCV RNA compared to aaU-HCV RNA synthesized with decreasing amounts of aaU. As the amount of aaU is decreased the migration of aaU-HCV RNA begins to approach that of HCV RNA. B) HPLC analysis displayed a reciprocal relationship between aaU and U as the concentration of aaU in the transcription reaction was decreased. X:Y = aaU:UTP in transcription reaction.

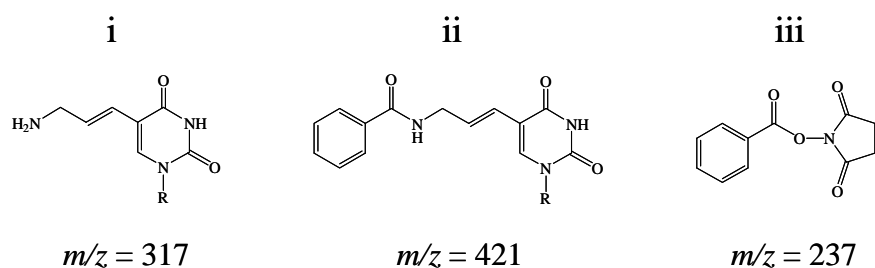


**Figure S2.** HPLC resolution of digested and dephosphorylated RNA. HCV RNA (top) clearly showed the presence of C, U, G and A. Analysis of aaU-HCV RNA (bottom) showed an additional eluent that corresponded to aaU. Samples were detected at 254 nm.

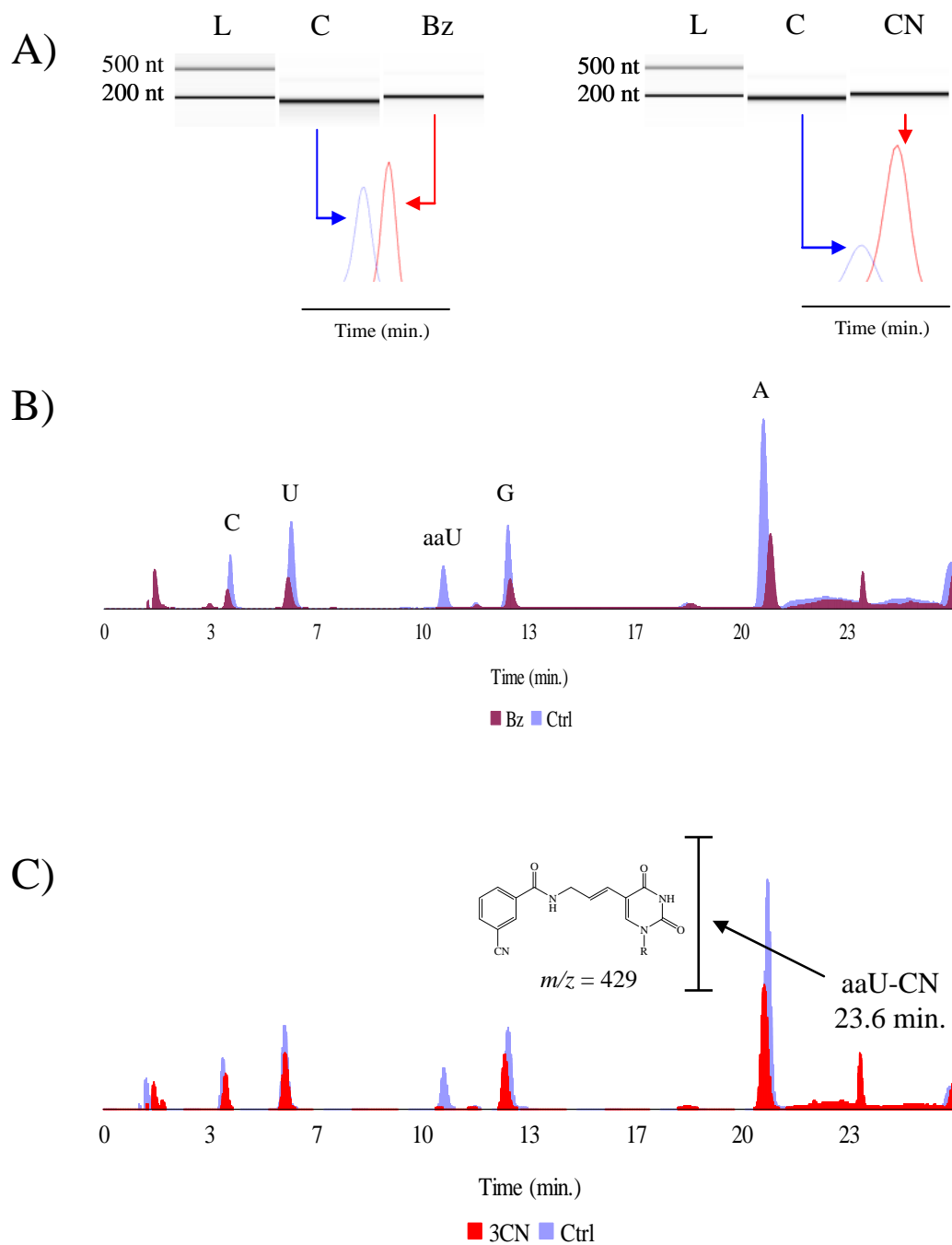
A)



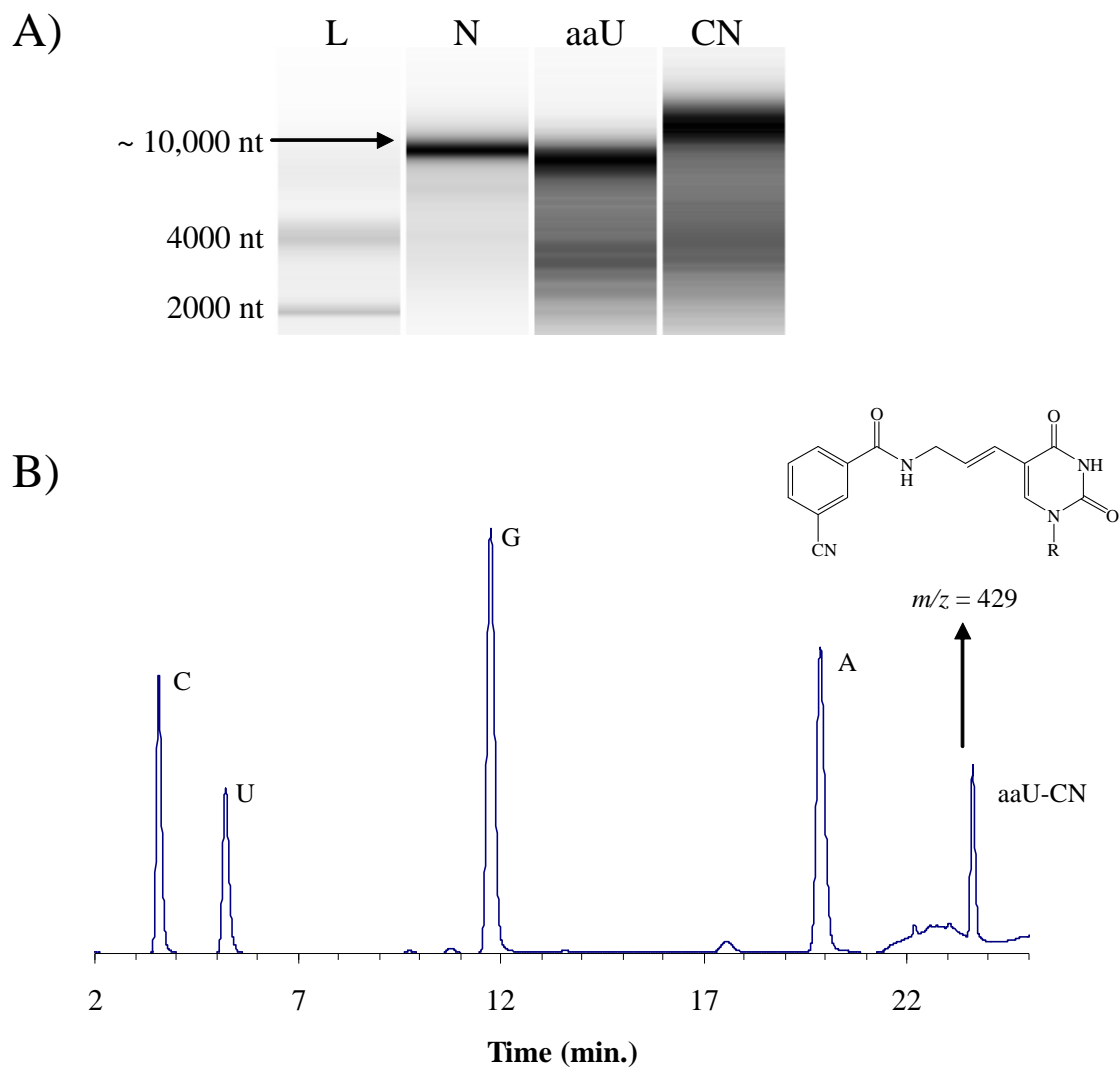
B)



**Figure S3.** Evaluating aaU modification with Bz-NHS. A) Bz-NHS eluted at 22.5 min. (blue). In the presence of aaU a new peak at 23.6 min. (pink) appeared, in addition to the Bz-NHS peak. This coincided with the disappearance of the aaU peak at 10.6 min. (green). B) Structures of aaU (**i**), aaU-Bz (**ii**) and Bz-NHS (**iii**). LC-MS confirmed the identity of the peaks corresponding to these materials. R = dephosphorylated ribose sugar.



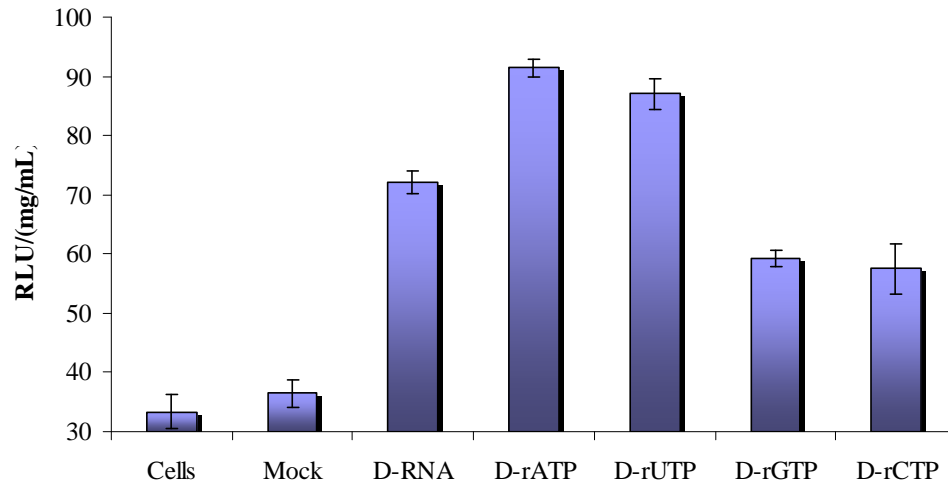
**Figure S4.** Labeling of a model RNA system with Bz-NHS and 3-CN-NHS. A) Electrophoretic migration of 163mer modified with Bz-NHS (left) and 3-CN-NHS (right). Labeled products showed an increase in apparent mass. B) HPLC showing the disappearance of aaU (10.6 min.) and the appearance of Bz-modified aaU (23.6 min.) when control (Ctrl, blue) and Bz-modified (Bz, pink) 163mer reactions were analyzed. C) HPLC comparison of 163mer control (Ctrl, blue) and 3-CN-NHS (3CN, red) labeling reactions showing the appearance of aaU-CN (23.6 min.) and corresponding disappearance of aaU (10.6 min.).



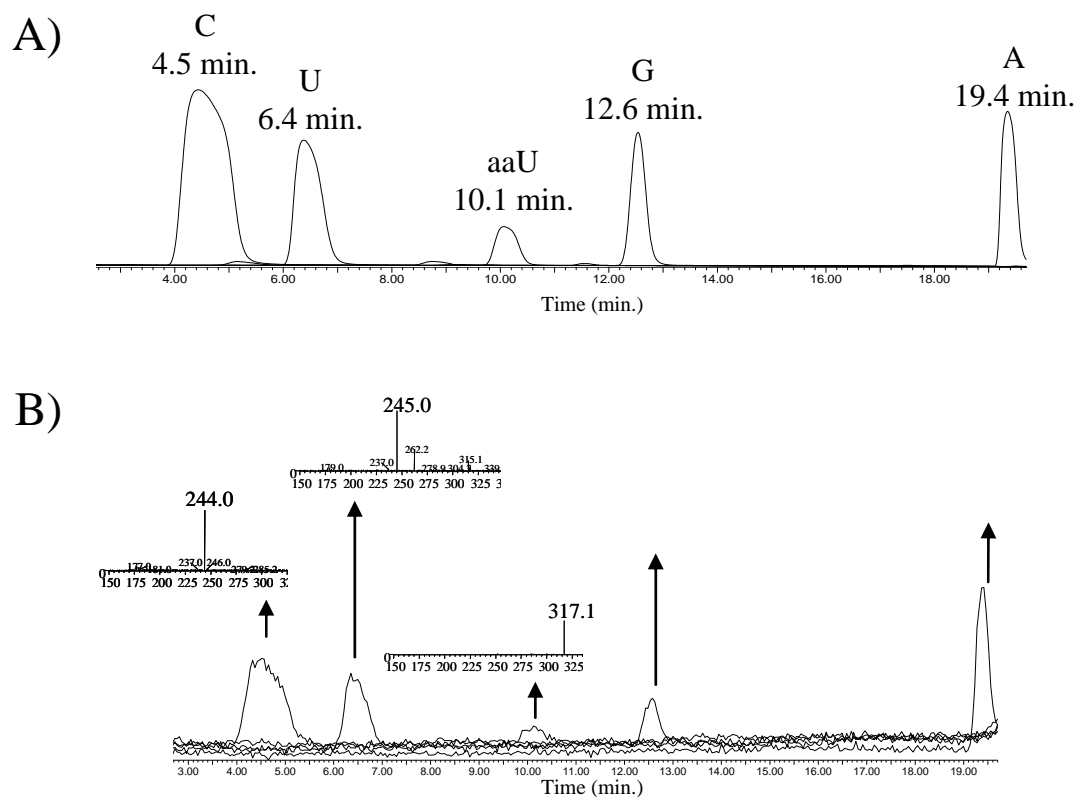


	Diameter (nm)	Spread (nm)
HCV RNA	30.7	17.7
aaU-HCV RNA	28.5	14.7
D-HCV RNA	24.3	10.0
CN-HCV RNA	31.6	17.5

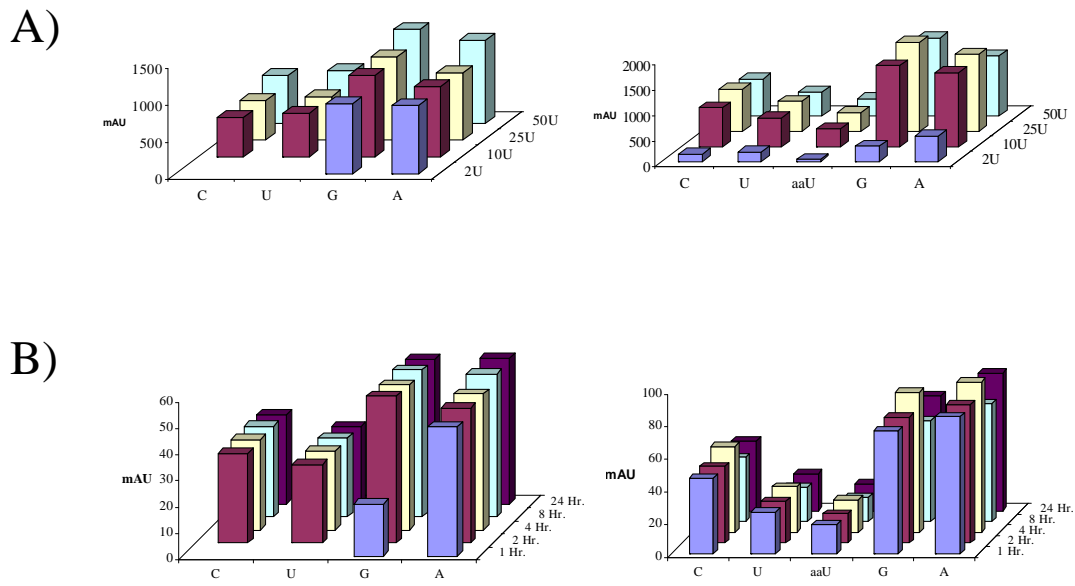
**Figure S6.** DLS analysis of modified HCV RNAs. No clear difference between the normal and modified HCV RNAs could be resolved using DLS. However, the size of the collapsed state approximated that required to fit inside the mature HCV virion (~30 nm). All values are reported as mean of 100 measurements. Diameter = mean hydrodynamic diameter; Spread = size of population distribution.



**Figure S7.** Decreases in luciferase activity were causally linked to the incorporation of specific D-rNTPs into HCV replicon RNA. Incorporation of deuterated ATP and UTP affected luciferase activity less than deuterated GTP and CTP. The reasons for this remain unclear. All measurements were made in triplicate and are reported as mean values  $\pm$  one standard deviation. D-RNA = D-HCV RNA



**Figure S8.** Optimizing the elution conditions of 5 mononucleosides. A) C, U, A, G and aaU were all resolved as shown by this overlay of their respective elution profiles. Samples were detected at 254 nm. B) C, U, A and G were identified by LC-MS as molecular ions (M+1) at a concentration of 10 mM. The modified uridine, aaU, was identified from a weekly ionizing ammonium adduct (M+18) at a concentration of 6 mM. Even after accounting for concentration differences the ionization of aaU was low.



**Figure S9.** Validating the digestion of HCV RNA and aaU-HCV RNA. A) The digestion reaction saturated between 2 – 10U for HCV RNA (left) and 10 – 25U for aaU-HCV RNA (right). B) The digestion of HCV RNA (left) and aaU-HCV RNA (right) saturated after 2 hours. C and U 2U (A) and 1 Hr. (B) data points were not collected.