# Supporting Information for:

# Synthesis and Biophysical Characterization of $tRNA^{Lys,3}$ Anticodon Stem-Loop RNAs Containing the $mcm^5s^2U$ Nucleoside.


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### Experimental Section

**General Methods.** Melting points are recorded by the open capillary method and are uncorrected. Standard nucleoside phosphoramidite procedures were used to convert the base-protected modified nucleosides to their respective 5'-DMT,-2'-TBS, 3'-phosphoramidites[1]. $^1$H and $^{31}$P NMR were collected in deuterated solvents on either 200 or 500 MHz spectrometers. $^1$H chemical shifts are reported relative to tetramethylsilane at 0.0 ppm and referenced to the residual proton signal of the deuterated solvents. $^{31}$P chemical shifts are relative to external phosphoric acid at 0.0 ppm. Analytical thin layer chromatography (TLC) was carried out on Merck Silica gel 60F-254 plates. Column chromatography was carried out with Merck silica gel 60 (230-400 mesh). Chemical reagents were purchased from Aldrich except where noted and used without further purification. Spectral grade acetonitrile (Baxter) was used for HPLC. Instrumentation and sample preparation for MALDI MS and ESI LC/MS are described below.

**5'-O-(Dimethoxytrityl)-5-carbomethoxymethyl-2-thiouridine** (**2a**): To a solution of 5-carbomethoxymethyl-2-thiouridine[2] (1.00 g, 3.01 mmol) in dry pyridine (30 mL) was added 4,4'-dimethoxytritylchloride (1.22 g, 3.61 mmol) at room temperature. The reaction mixture was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure, and the brown residue was co-evaporated with toluene (2 x 25 mL). The crude material was purified by silica gel chromatography using mixture of dichloromethane and methanol (9:1) to gave **2a** (1.53 g, 80 %) as a colorless solid: mp 120-123 ºC; $^1$H NMR (Acetone – d6) δ 2.84 (s , 2H), 3.48 –3.56 (m, 2H), 3.57 (s, 3H), 3.78 (s, 6H) 4.20 (d, J = 5.81 Hz,1H), 4.30 – 4.55 (m, 3H), 4.78 – 4.89 (m, 1H), 6.72 (d, J = 3.83 Hz, 1H), 6.84 – 6.92 (m, 4H), 7.24 –7.48 (m, 9H), 7.98 (s, 1H), 11.35 (s, 1H); HRMS (CI) MH+ calcd for $C_{33}H_{35}N_2O_9S$: 635.2063, obsd 635.2008. Anal. Calcd for $C_{33}H_{35}N_2O_9S$: C, 62.44; H, 5. 39; N, 4.41. Found: C, 62.22; H, 5.20; N, 4.30.

**5'-O- (4,4'-Dimethoxytrityl)-5-carbomethoxymethyluridine** (**2b**). Compound **2b** was prepared from 5-carbomethoxymethyluridine[2] (1.00 g, 3.16 mmol) according to the procedure described for **2a**, Yield. 1.33 g (68 %): mp 126-129 ºC; $^1$H NMR (Acetone – d6) δ 2.61 (s, 2H), 3.84 –

3.41 (m, 2H), 3.55 (s, 3H), 4.11 (d, J = 4.20 Hz, 1H), 4.30 – 4 .50 (m, 3H), 4.66 – 4.82 (m, 1H), 5.10 (d, J = 3.82 Hz, 1H), 6.86 – 7.01( m, 4H), 7.23 – 7.49 (m, 9H), 7.74 (s, 1H), 10.15 (s, 1H); HRMS(CI) MH+ calcd for $C_{33}H_{35}N_2O_{10}$: 619.2291, obsd 619.2274. Anal. Calcd for $C_{33}H_{35}N_2O_{10}$: C, 64. 07; H, 5.53; N, 4.52. Found: C, 63. 89; H, 5.72; N, 4.36.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)-5-carbomethoxymethyl 2-thiouridine (3a)**. A solution of **2a** (1.50 g, 2.36 mmol) was dissolved in dry pyridine (15 mL) under nitrogen atmosphere. To this were added imidazole (0.64 g, 9.45 mmol) and tert-butyldimethylsilyl chloride (0.43 g, 2.84 mmol). The reaction mixture was stirred at room temperature for 10 h. The solvent was evaporated under reduced pressure and the residue was co-evaporated with toluene (30 mL x 2). The pale yellow residue was dissolved in dichloromethane (100 mL) and washed with 5% aqueous sodium hydrogen carbonate (2 x 50 mL) followed by water (50 mL), dried over $Na_2SO_4$, filtered and the solvent then evaporated under reduced pressure to gave a mixture of 2' and 3' isomers. The crude mixture of 2' and 3' isomers was separated by flash chromatography over silica gel using a mixture of dichloromethane and ethyl acetate (9:1) to afford the isolated 2'- isomer (0.71 g, 40 %) and 3'-isomer (0.69 g, 39 %). To a methanolic solution of the 3' isomer was added a trace of triethylamine and the solution stirred at RT to give mixture of 2' and 3' isomers. The isomerization and chromatography was repeated two times to obtain an additional quantity of 2' isomer for an overall yield of 1.22 g (69 %): mp 105 -106 °C; $^1$H NMR (Acetone–d6) δ 0.16 (s, 6H), 0.94 (s, 9H), 2.81 (s, 2H), 3.46 –3.49 (m, 2H), 3.55 (s, 3H), 3.78 (s, 6H), 4.11 (d, J = 4.80 Hz, 1H), 4.15 – 4.30 (m, 1H), 4.40 – 4.44 (m, 1H), 4.57 (t, J = 4.60 Hz, 1H), 6.65 – 6.92 (m, 5H), 7.72 –7.47 (m, 9H), 7.98 (s, 1H), 11.40 (s, 1H); HRMS (CI) MH+ calcd for $C_{39}H_{49}N_2O_9SiS$: 749.2928, obsd 749.2933. Anal. Calcd for $C_{39}H_{49}N_2O_9SiS$: C, 62. 54; H, 6.45; N, 3.74. Found: C, 62.38; H, 6.39; N, 3.56.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)-5-carbomethoxymethyluridine (3 b )**. Compound **3b** was prepared from 5'-O-(4,4'-Dimethoxytrityl)-5-carbomethoxymethyluridine (**2b)** (1.3 g, 2.10 mmol) according to the procedure described for **3a**.Yield. 1.28 g (83 %): mp 118-119 °C; $^1$H NMR (Acetone - d6) δ 0.16 (s, 3H), 0.17 (s, 3H), 0.9 (s, 9H), 2.82 (s, 2H), 3.41- 3.44 (m,2H), 3.42 (s,3H), 3.78 (s, 6H), 3.91 (d, J = 6.00 Hz, 1H), 4.00 – 4.18 (m, 1H), 4.34 – 4. 39 (m, 1H), 4.54 (t, J = 5.20 Hz, 1H), 6.03 (d, J = 5.20 Hz, 1H), 6.67 – 6.91 (m, 4H), 7.28 –7.47 (m, 9H), 7.77 (s,1H), 10.16 (s, 1H); HRMS (CI) MH+ calcd for $C_{39}H_{49}N_2O_{10}S$: 733.3156, obsd 733.3121. Anal. Calcd for $C_{39}H_{49}N_2O_{10}S$: C, 63.91; H, 6.00; N, 3.82. Found: C, 64.01; H, 6. 21; N, 3.82.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)-5'-carbomethoxymethyl-2-thiouridine-3'-(cyanoethyl-N,N-diisopropylphosphoramidite) (4a).** Compound **3a** ( 0.25 g, 0.33 mmol ) was dissolved in dry THF (15 mL) under argon atmosphere. To this was added DMAP (8.1 mg, 0.06 mmol), and diisopropylethylamine (114 μL, 0.66 mmol). To the above reaction mixture 2-cyanoethyl-N,N-diisopropyl chlorophosphoramidite (148 μL, 0.665 mmol ) was added with stirring. After 30 min a white solid formed and the stirring was continued for an additional 3 h. The reaction was followed by taking a 50 μL of aliquot of the reaction mixture, removing the solvent, oxidizing for 3 min with tBuOOH/toluene, evaporating the toluene, and then analyzing by silica gel TLC. The oxidized amidite product is found at the origin while unreacted product is unaffected by the oxidation procedure. This protocol is helpful since the

starting material and the products migrate with identical RF in the solvent systems that we investigated. The reaction mixture was extracted with ethyl acetate ( 50 mL), washed with 5% sodium bicarbonate (25 mL) and finally with water (2 x 25 mL). The organic layer was dried over $Na_2S_2O_4$, filtered, and the solvent evaporated under reduced pressure. The residue was purified by flash chromatography over silica gel using dichloromethane and ethyl acetate (9:1). to yield **4a** (0.23 g, 75 % ). The material exists as 1:1 ratio of stereoisomers about phosphorous and two chemical shift were observed for some of the resonances. The secondary shifts are indicated in parenthesis: mp 92 – 93 °C; $^1$H NMR (Acetone – d6) δ 0.15 – 0.28 (m), 0.93 (0.96) (s, 9H), 1.04 –1.30 (m), 2.50 –2.62 (m), 2.72 –2.90 (m), 3.40 –3.51 (m), 3.55 (3.56) (s, 3H), 3.58 – 3.74 (m), 3.78 (s, 6H), 3.90 – 4.10 (m), 4.30 – 4.48 (m), 4.62 – 4.75 (m), 6.68 – 6.93 (m, 5H), 7.27 –7 .45 (m, 9H), 7.94 (7.96) (s, 1H), 11.40 (s, 1H); $^{31}$P NMR (Acetone – d6) δ 154.78, 156.19; MS(FAB) calcd for $C_{48}H_{65}N_4O_{10}SiSP$: 949.19, obsd 949.50. Anal. Calcd for $C_{48}H_{65}N_4O_{10}SiSP$: C, 60. 73; H, 6.90; N, 5.90. Found: 60.92; H, 6.80; N, 5.77.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)-5'-carbomethoxymethyluridine-3'-(cyanoethyl-N,N-diisopropylphosphoramidite) (4b).** Compound **4b** was prepared from 5'-O-(4,4'-dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)-5-carbomethoxymethyluridine (**3b**) (0.25 g, 0.34 mmol) according to the procedure described for **4a**. The material exists as 1:1 ratio of stereoisomers about phosphorus and two chemical shifts were observed for some of the resonances. The secondary shifts were indicated in parenthesis, Yield 0.28 g ( 88 %): mp 96-98 °C; $^1$H NMR (Acetone – d6) δ 0.08 -.122 (m), 0.91(0.94) (s, 9H), 1.04 –1.35 (m), 2.5 – 2.60 (m), 2.76 –2.81 (m), 3.42 – 3.74 (m), 3.78 (s, 6H), 3.92 – 4.61(m), 6.04 – 6.11 (m), 6.67 –6.93 (m, 4H), 7.26 –7.46 (m, 9H), 7.78 (s, 1H); $^{31}$P NMR (Acetone – d6) δ 149.56, 151.75; MS(CI) calcd for $C_{48}H_{65}N_4O_{11}SiP$: 933.12, obsd 933.50. Anal. Calcd for $C_{48}H_{65}N_4O_{11}SiP$: C, 61.78; H, 7.02; N, 6.00. Found: C, 60.40; H, 6.67; N, 5.34.

**Oligonucleotide Synthesis**. The oligonucleotides were synthesized on an Applied Biosystems 394 oligonucleotide synthesizer on a 1 μmole scale using 0.05 M acetonitrile solutions of PAC amidites (PAC A, isopropyl PAC G, and acetyl C) from Glen Research. The concentrations of $mcm^5s^2U$, and $mcm^5U$ amidites were 0.12 M each. The phosphoramidites were coupled for 25 min. The normal $I_2/H_2O$ oxidation solution was used until the $mcm^5s^2U$ nucleoside and then 2 x 5 min oxidation cycles with 10% tBuOOH in acetonitrile was used from that point forward.

**RNA Deprotection and Purification**. The CPG-bound RNA sequences were transferred from the column to a screw cap glass vial, to which was added 1 mL of 10 % DBU in methanol[3]. The solution was stirred at room temperature for 10 h. The supernatant was decanted, the support was washed with additional 1.0 mL of 10% DBU in methanol, and the combined washings were lyophilized on a Speed-Vac concentrator. The dried material was dissolved in 1mL neat $Et_3N.3HF$ (Aldrich) and stirred at room temperature for 9 -12 h[4,5]. The reaction was quenched by adding 0.1 mL of water, and the RNA was precipitated by adding 10 mL of n-butanol and allowing the solution to stand at –20 °C for 6h . After centrifugation, the RNA pellet was dissolved in 0.5 mL of water and purified by anion exchange HPLC[6]. The fractions containing full length material as judged by HPLC were collected, lyophilized, and then dialyzed against 2 x 1 L of deionized water. The dialyzed material was lyophilized in preparation for further analysis.

**Sample Preparation for MALDI**. All matrix compounds used in this work were purchased from Sigma Chemical Co. and were used without further purification. Matrix and co-matrix solutions were prepared as follows. 0.04 g of 6-aza-2-thiothymine (ATT matrix) was added to 500 $\mu$L of 50 % acetonitrile and mixed on a Vortex mixer for 30 s. Ammonium citrate co-matrix solution was prepared by dissolving 0.023 g of ammonium citrate in 1 mL of water. Oligonucleotide concentrations for most samples were 10 pmol/μL. For each spectrum, 0.5 $\mu$L of the sample solution was spotted onto a gold plated sample well and allowed to air-dry. A 0.5 $\mu$L aliquot of co-matrix solution was added to the dry sample and allowed to air-dry. A 0.5 $\mu$L aliquot of the matrix solution was then added to the sample/co-matrix mixture in the sample well. After the sample had again dried, the MALDI probe was inserted into the mass spectrometer.

**MALDI mass Spectrometry**. MALDI spectra were obtained on a reflectron MALDI/TOF mass spectrometer (Model: Perseptive Voyager-DE STR, PE Applied Biosystems Co., Foster City, CA) equipped with a delayed extraction ion source, and a pulsed linear detector. A nitrogen laser at 337 nm (3 ns pulse width) was used to desorb the ions in the source region. The time-of-flight data were either externally calibrated or mass converted using ion peaks of known masses. Background pressure within the instrument was less than 1x $10^{-7}$ Torr as measured by a Bayard-Alpert ion gauge located below the source.

**Combined Liquid Chromatography / Mass Spectrometry (LC/MS) Analysis of the Nucleoside Content of Oligonucleotide**. The $tRNA^{Lys,3}$-$mcm^5s^2U$, ψ and $tRNA^{Lys,3}$ $mcm^5U$,ψ oligonucleotides were digested to nucleosides using nuclease P1, phosphodiesterase I, and alkaline phosphatase[7,8]. The resulting nucleoside mixture was analyzed by LC/MS using a HP 1090 liquid chromatograph interfaced to a Fisons Quattro II triple quadrupole mass spectrometer (Manchester, U.K.) using electrospray ionization. As shown in Figures **S8** and **S9,** the HPLC elution monitored at 260 nm indicates that RNA oligonucleotides contains the 4 major nucleosides as well ψ (4.09 min), $mcm^5s^2U$ (25.68 min) and $mcm^5U$ (18.55 min). The six nucleosides in the sample elute with correct retention times and their identities were verified by mass spectrometry as they eluted.

## References for Supporting Information:


(1) Scaringe, S. A.; Francklyn, C.; Usman, N. *Nucleic Acids Res*. **1990**, *18*, 5433-5441.
(2) Fisseskis. J. D.; Sweet. F. *Biochemistry,* **1970**, 9 (16). 3136.
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(5) Gasparutto, D.; Livache,T,; Bazin,H.; Dupla,A.; Guy,A.; Khordin,A.; Molko,D.; Roget,A.; Teoule,R. *Nucleic Acid Res*. **1992**, 20, 5159.
(6) Sproat,B.; Colonna, F.; Mulla, B.; Tsuo, D.; Andrus, A.; Hampel, A.; Vinayaka, R. *Nucleosides and Nucleotides,* **1995**, 14, 255.
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(8) Crain, P, F. *Methods Enzymol,* **1990**, 193, 782.

**Legends for Figures S1-S9.**

**Figures S1-S4.** $^{1}$H NMR spectra for compounds **2b, 3b, 3a, 4a.**

**Figure S5.** Ion-exchange HPLC of the $tRNA^{Lys,3}$ stem loops. HPLC was done at 60 $^{o}$C Using a 1 x 15 cm Pharmacia Resources Q column. The mobile phase was 0.02 M ammonium acetate and the RNA was eluted with a gradient of 20-600 mM $LiClO_4$. The Figure shows analytical traces of HPLC purified RNA oligonucleotides **(a)** $tRNA^{Lys,3}$-$mcm^5s^2U34$,ψ 39; **(b)** $tRNA^{Lys,3}$-$mcm^5U34$,ψ39.

**Figure S6.** MALDI mass spectrum of the $tRNA^{Lys,3}$ $mcm^5s^2U34$, ψ39. The spectrum is shown for HPLC purified RNA that had been ethanol precipitated from 10 M $NH_4OAc$ to decrease sodium ion adducts. Calculated molecular weight ($MH^+$) 5421.36; found 5421.

**Figure S7.** MALDI mass spectrum of the $tRNA^{Lys,3}$-$mcm^5U34$, ψ39. The spectrum is shown for HPLC purified RNA that had been ethanol precipitated from 10 M $NH_4OAc$ to decrease sodium ion adduction. Calculated molecular weight ($MH^+$) 5404. 3; Found 5408.

**Figure S8**. Electrospray ionization LC/MS analysis of the enzymatically digested $tRNA^{Lys,3}$-$mcm^5s^2U34$, ψ39. The top panel is the UV (260 nm) monitored HPLC trace of the enzyme digest showing the elution of the 4 major nucleosides along with ψ (4.09), and $mcm^5s^2U$ (25.68). Panels **B-F** show selected ion profiles representing the molecular masses (MH+) for each nucleoside. In addition, complete mass spectra were collected for each eluting nucleoside( not shown ). The molecular ion masses are indicated in each of the MS panels and correctly match appropriate MH+ values of the major, and the 2 modified nucleosides. (**B**) ψ ( 4.09) and uridine (5.90) (early eluting materials from the digest contribute to the MS in this panel); (**C**) cytidine; (**D**) guanosine; (**E**) adenosine; (**F**) $mcm^5s^2U$.

**Figure S9**. Electrospray ionization LC/MS analysis of the enzymatically digested $tRNA^{Lys,3}$-$mcm^5U34$, ψ39. The top panel is the UV (260 nm) monitored HPLC trace of the enzyme digest showing the elution of the 4 major nucleosides along with Ψ (4.08), $mcm^5U$ (18.55). Panels **B-F** show selected ion profiles representing the molecular masses (MH+) for each nucleoside. In addition, complete mass spectra were collected for each eluting nucleoside (not shown). The molecular ion masses are indicated in each of the MS panel and correctly match appropriate MH+ values of the major, and the 2 modified nucleosides. (**B**) ψ (4.08) and uridine (6.05) (early eluting materials from the digest contribute to the MS in this panel); (**C**) cytidine; (**D**) guanosine; (**E**) $mcm^5U$; (**F**) adenosine.

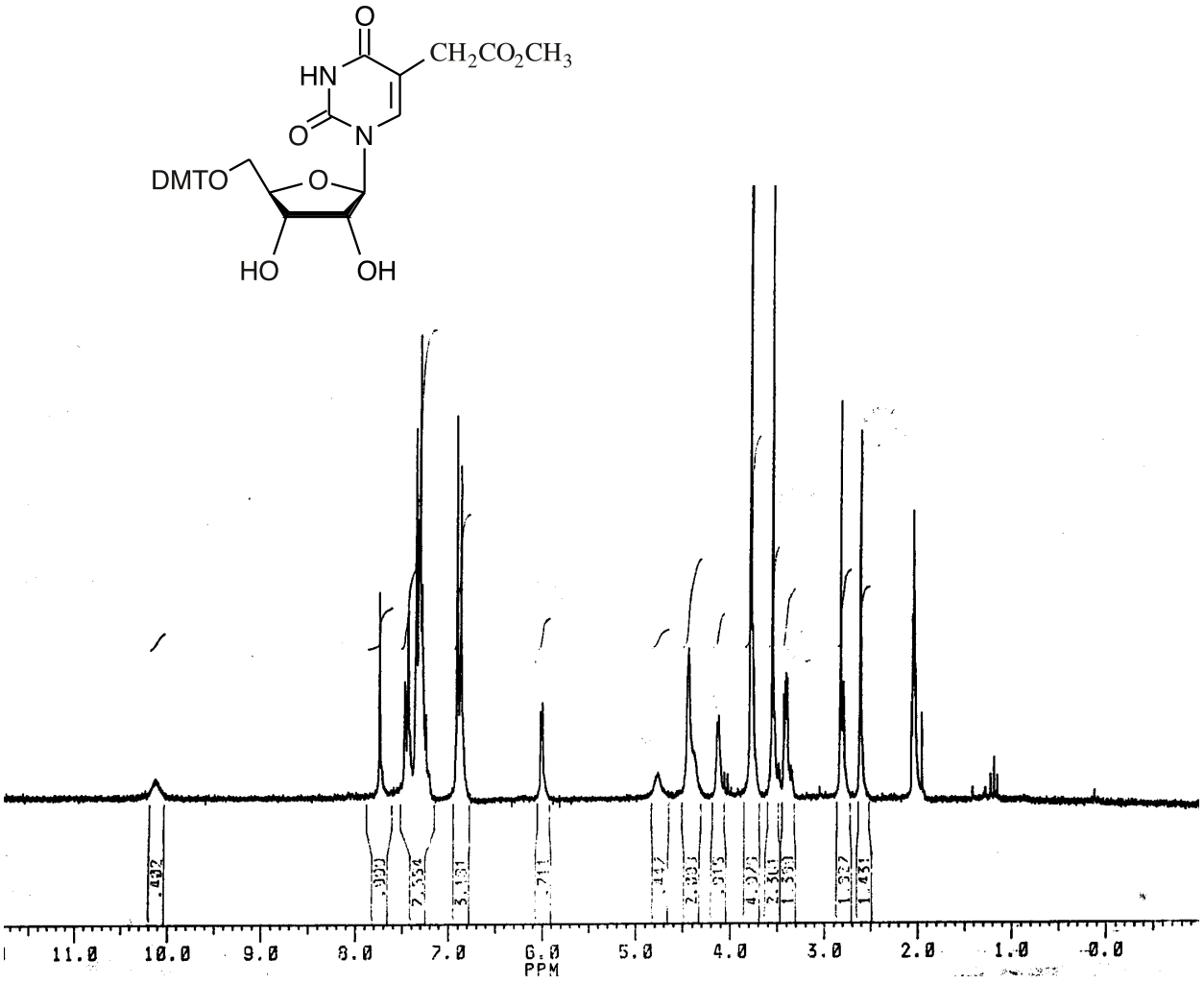
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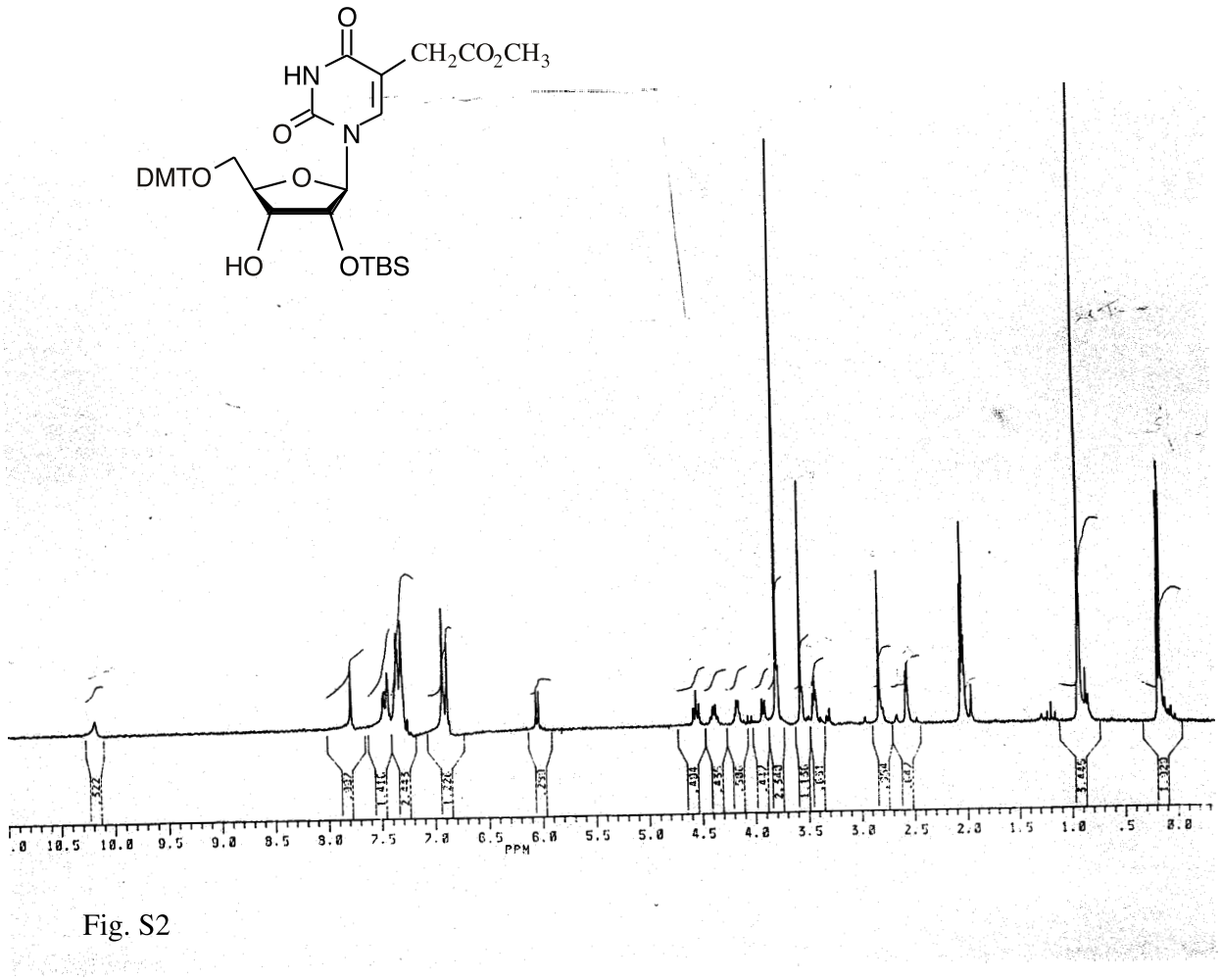
Fig. S1.

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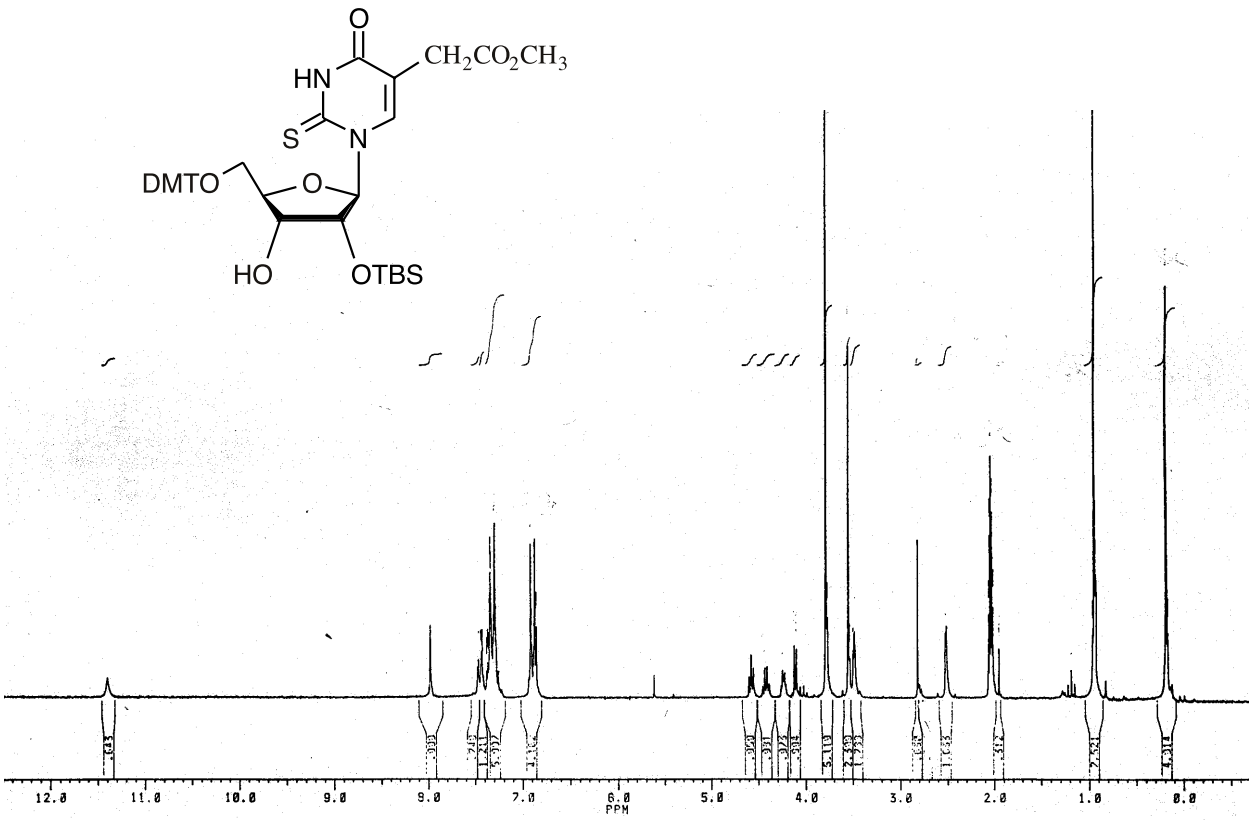
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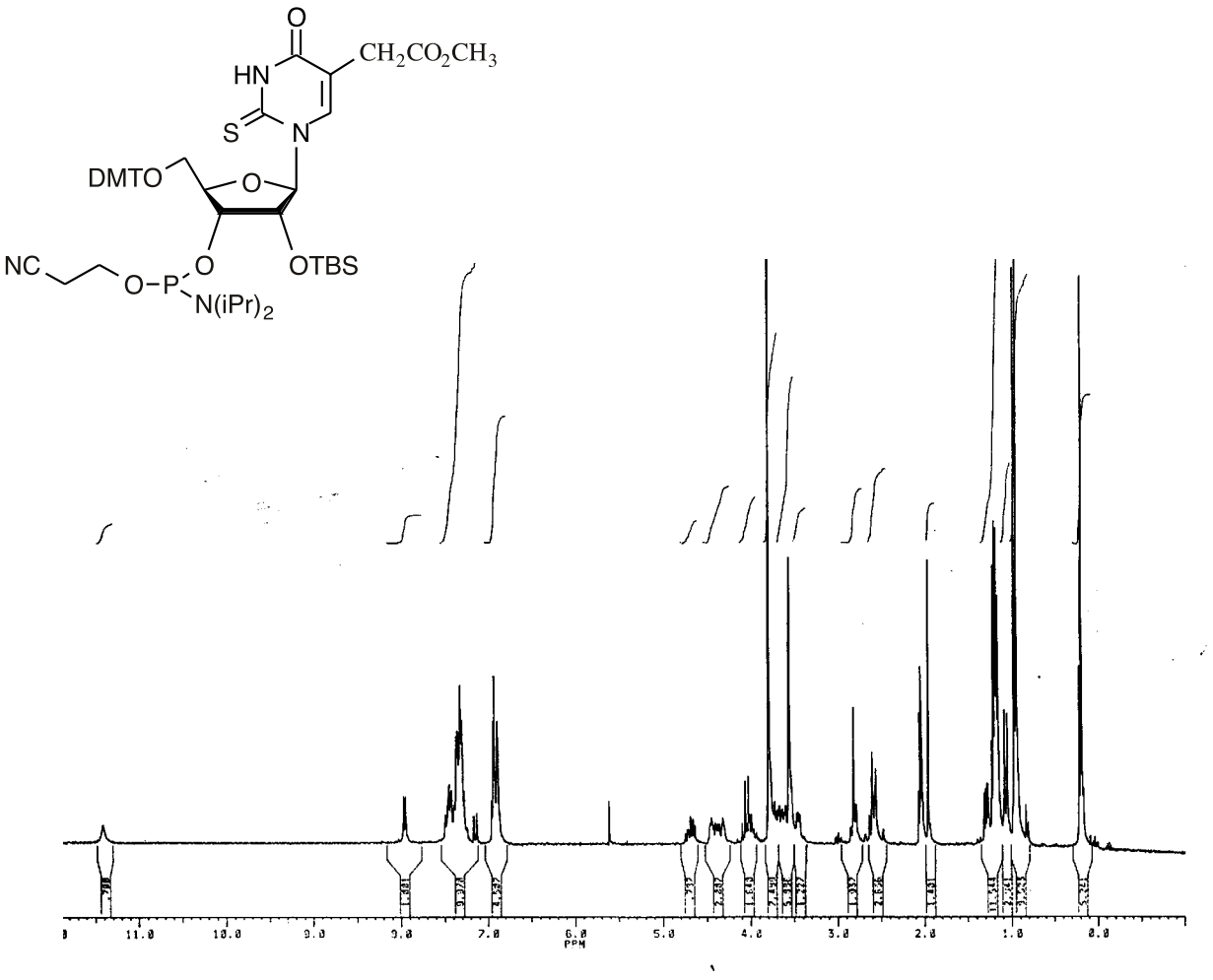
Fig. S3

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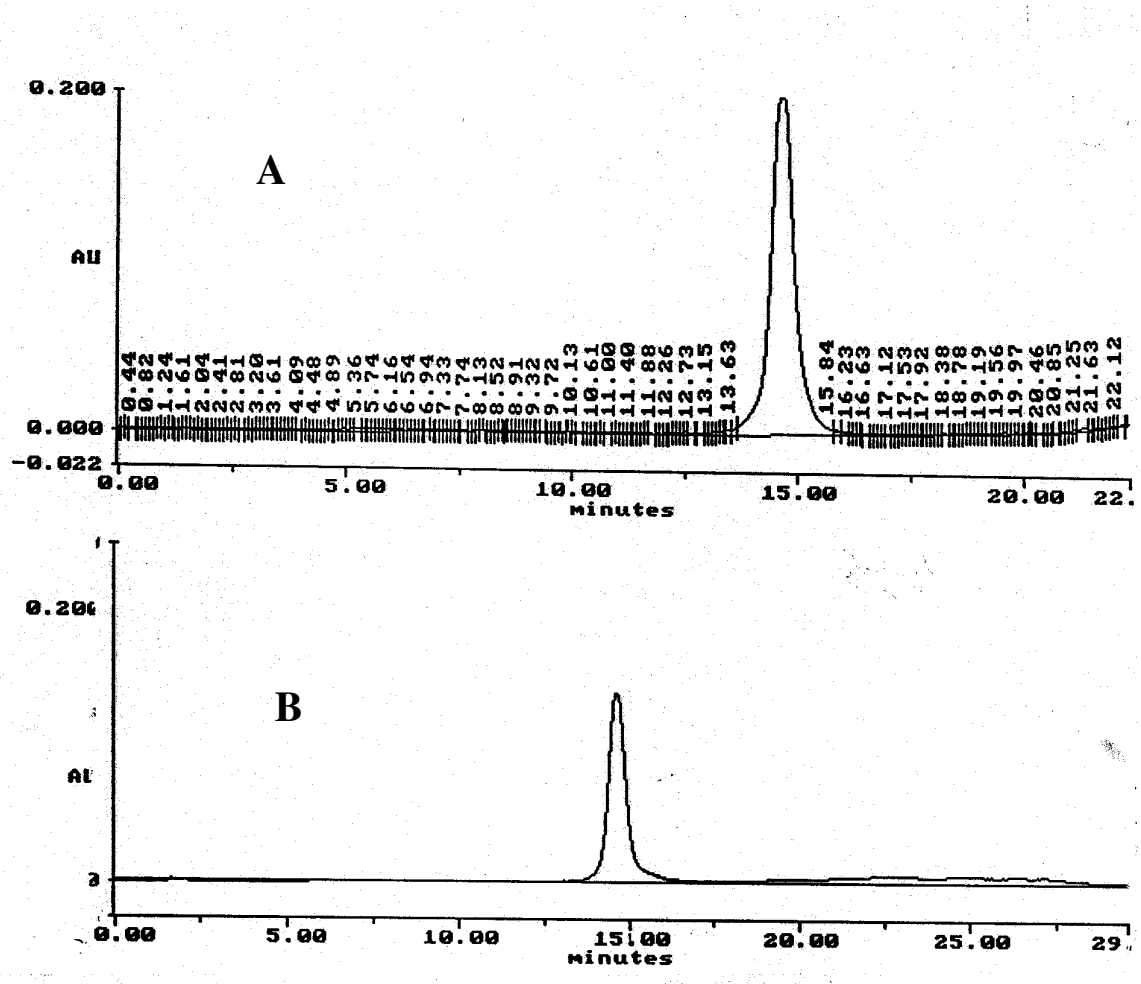
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Fig. S5.

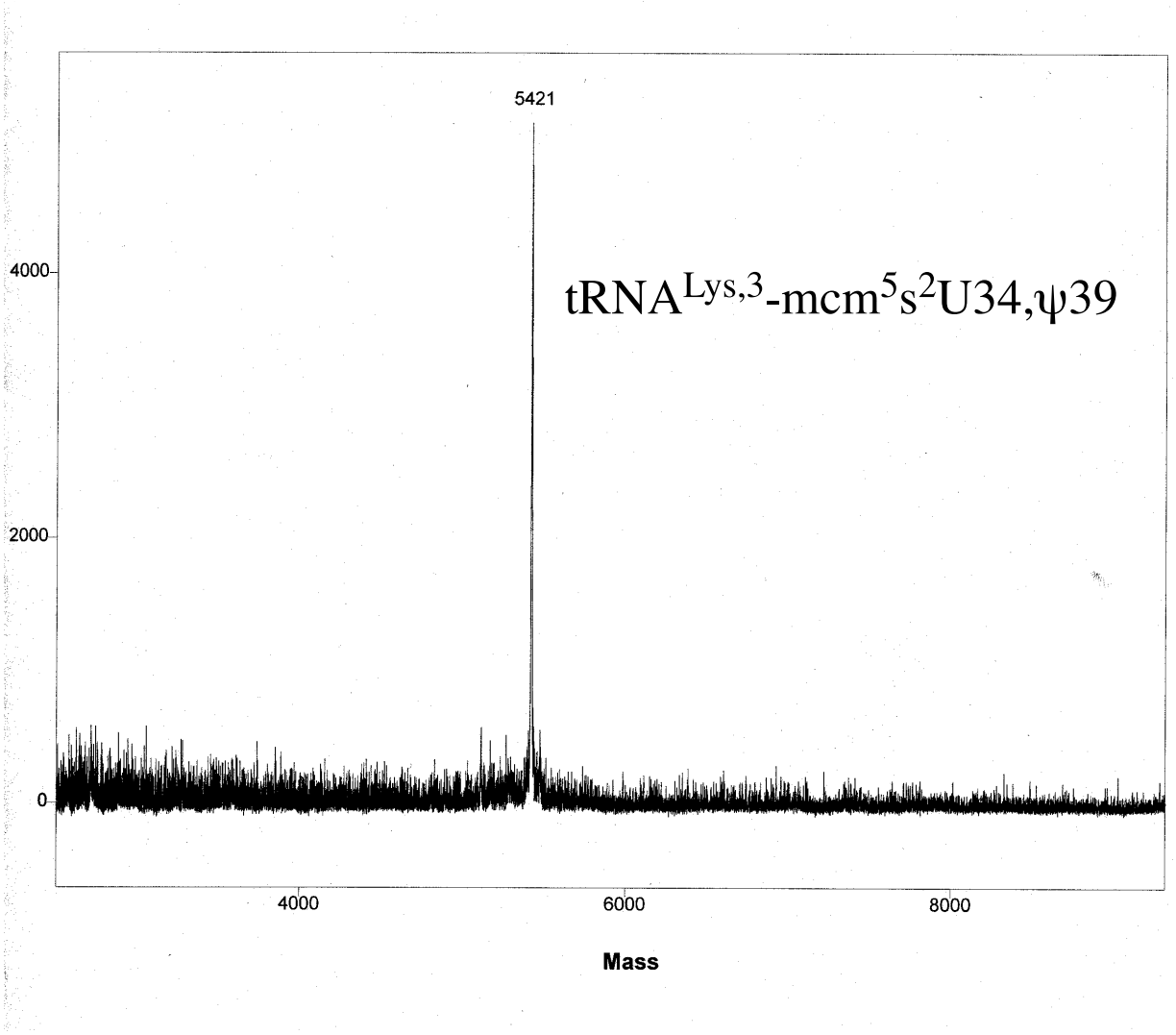


Fig. S6

$tRNA^{Lys,3}$-$mcm^5U34,\psi39$

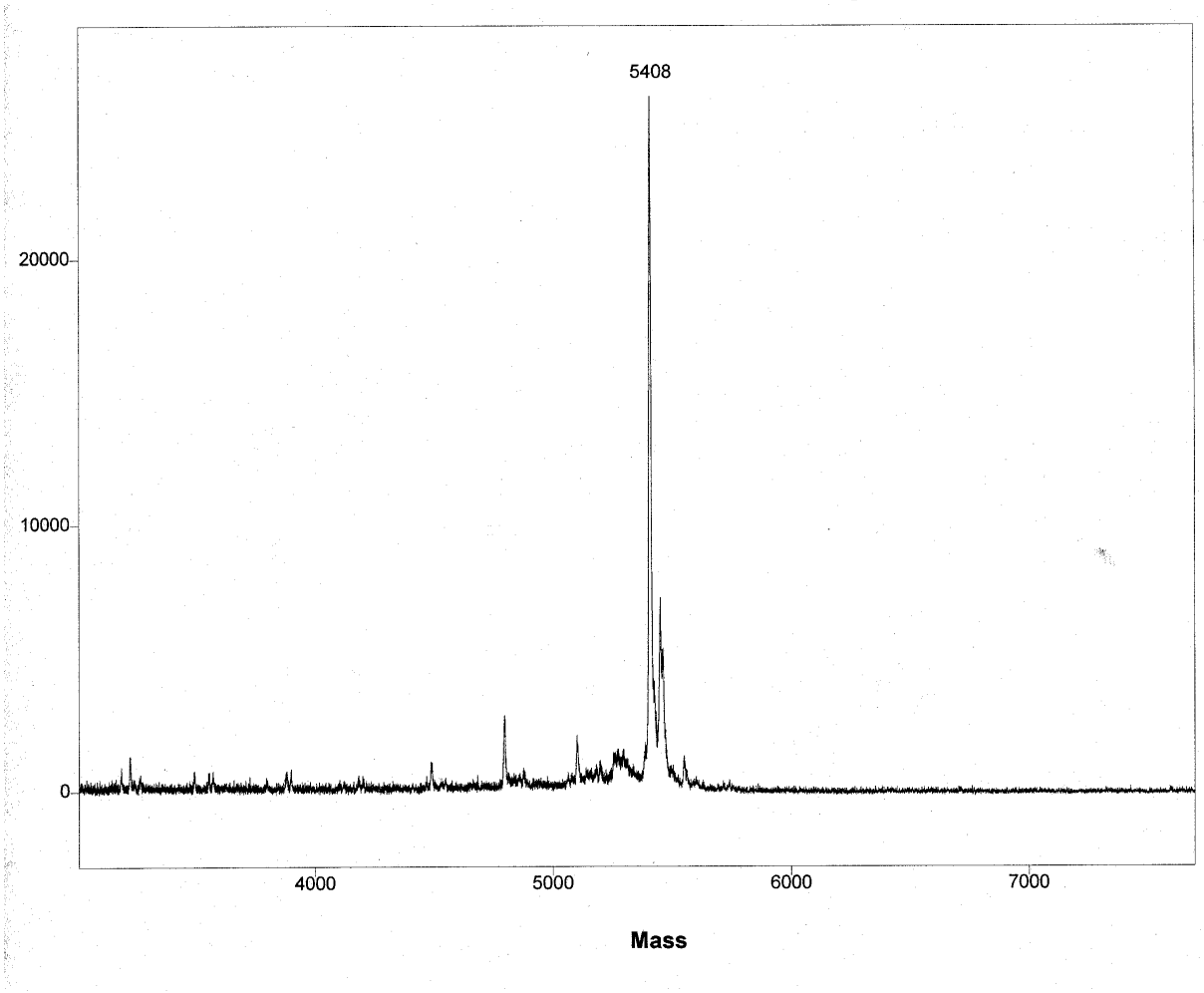


Fig. S7

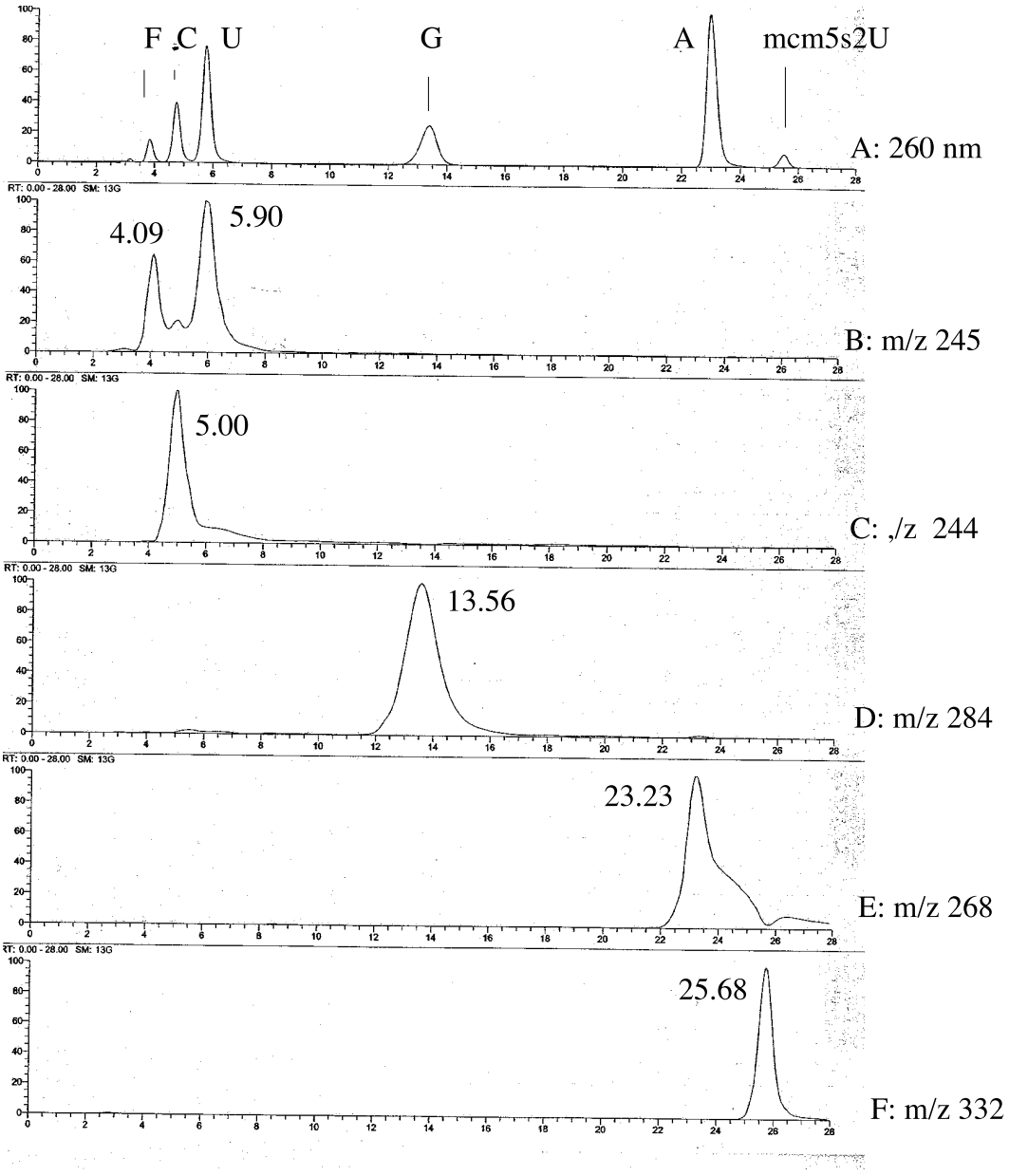
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Fig. S8

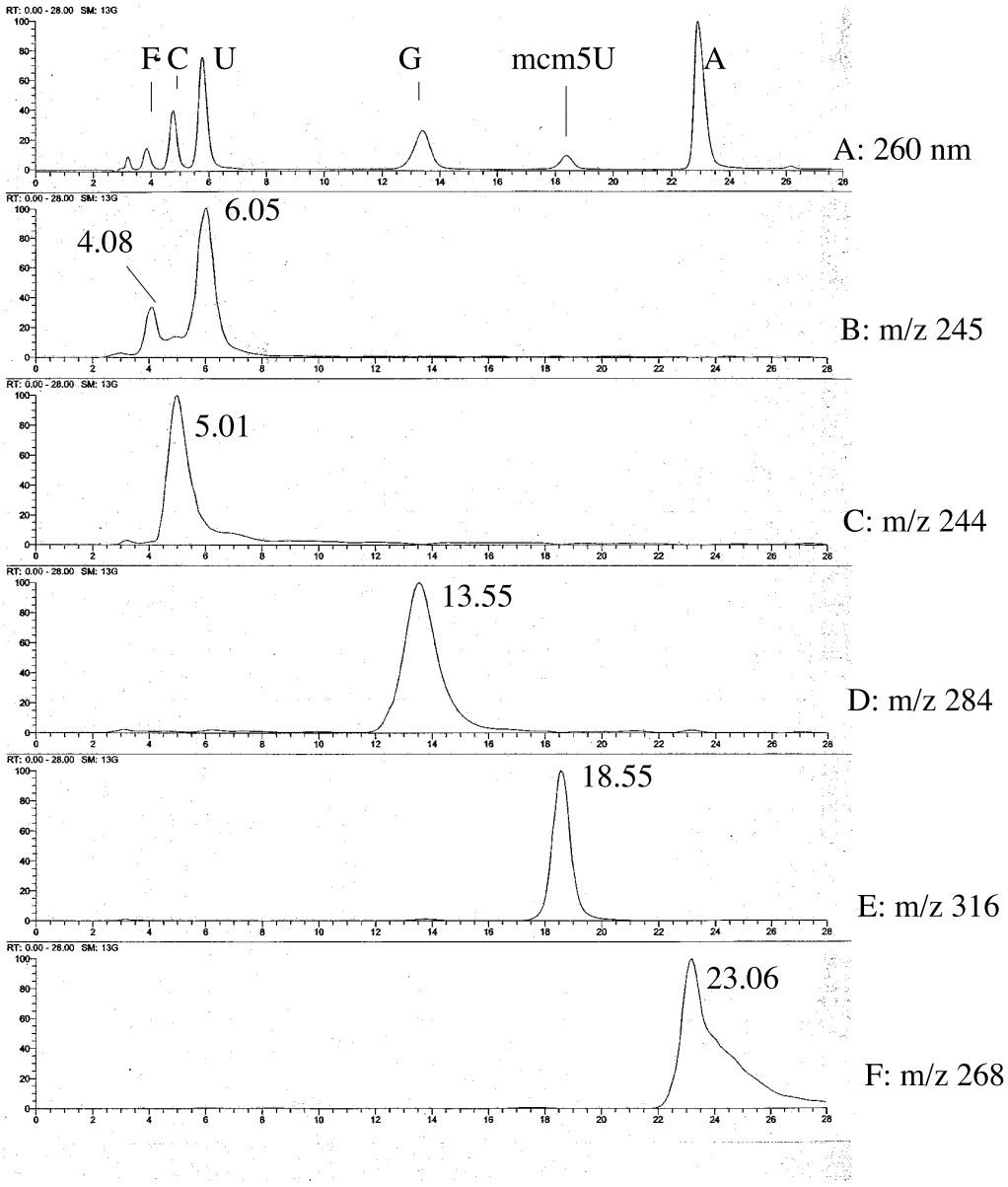
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Fig. S9