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## 1. General materials and methods.

**1.1. General materials.** Unless otherwise stated, all general chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or New England Biolabs (Ipswich, MA, USA). *E. coli* BL21 (DE3) competent cells were purchased from Invitrogen (Carlsbad, CA). The pET-28a *E. coli* expression vector and thrombin were purchased from Novagen (Madison, WI). Primers were purchased from Integrated DNA Technology (Coralville, IA). Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). Ni-NTA superflow column and gel filtration column HiLoad 16/600 were purchased from GE Healthcare (Piscataway, NJ). Amicon® Ultra centrifugal filter units were purchased from EMD Millipore (Merck, KGaA, Darmstadt, Germany). All other chemicals were reagent grade or better and purchased from Sigma (St. Louis, MO).

## 2. Cloning, expression and purification

**2.1 Cloning and expression:** The sequence of the genes encoding the enzymes involved in this study can be found within the National Center for Biotechnology Information (NCBI): *Actinomadura melliaura atmS8* (genebank accession number ABC02801.1), *atmS9* (genebank accession number ABC02802.1), *atmS14* (genebank accession number ABC02799.1) and *Micromonospora echinospora calS9* (genebank accession number AAM70333.1). Genes *atmS8* and *atmS9* were amplified [using PCR primer sets S8 forward 5'-ATACTGCATATGGTGCGTTTTCTAGGGGACGGCGA-3' and S8 reverse 5'-CAGTATGAATTCTCACCGCCCGATCCCGCGGTAGG-3'; S9 forward 5'-ATACTGCATATGACCGGACGTGTGGTGGTCACGGGC-3' and S9 reverse 5'-CAGTATGAATTCTCATTGGAGCCCGTTTTCCGGCAG-3', respectively) with the AT2433 producer *Actinomadura melliaura* genomic DNA as the template. The *calS9* gene was amplified [using PCR primer sets 5'-AATTCCATATGCCCAGATCCCTGGT-3' and reverse 5'-AATTCGAATTCCTACCTGACGACC-3'] with *Micromonospora echinospora* genomic DNA as template. The *atmS14* gene was amplified (using PCR primers S14 forward 5'-ATACTGCATATGCTGAAGCGACCTATCAGCGT-3' and S14 reverse 5'-CAGTATGAATTCTCACCAGAGTGTCTGCAGGCA-3') from a pUC57-based plasmid template containing a synthesized codon-optimized *atmS14* gene (Genscript, NJ; **Figure S1**) containing NdeI (forward) and EcoRI (reverse) restriction sites. The PCR products were digested with NdeI/EcoRI and ligated into suitably digested pET28a vector to provide expression plasmids for fusion proteins bearing the *N*-terminal-$His_6$ affinity purification tag. DNA sequencing was conducted by the University of Wisconsin Biotechnology Center with the primers T7 promoter (5'-TAATACGACTCACTATAGGG) or T7 terminator (5'-GCTAGTTATTGCTCAGCGG). The corresponding $His_6$-fusion proteins were overproduced in *E. coli* BL21(DE3) in the presence of 50 μg $mL^{-1}$ of kanamycin at 37 °C to an $OD_{600}$ of ~0.6, at which point the temperature was lowered to 25 ºC and gene expression induced with 0.5 mM IPTG. Cells were allowed to continue to grow at 25 ºC for approximately 18 hr at 220 rpm.

**2.2 Protein purification:** The cells were harvested by centrifugation (6,000 x*g*, 4 °C, 20 min) and resuspended in buffer A (20 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 7.8). The cells were lysed via incubation with 1 mg $mL^{-1}$ lysozyme (~50,000 U $mg^{-1}$; Sigma-Aldrich, St. Louis, MO, USA) for 30 min on ice followed by sonication (VirSonic 475; Virtis, Gardiner, NY; 100 W, 4 x 30 s pulses, ~1 min between pulses) on ice. Cell debris were removed by centrifugation (10,000 xg at 4 °C for 45 min) and purified via affinity chromatography (5 mL HiTrap Ni-NTA chelating column, GE Healthcare, Piscataway, NJ) using a standard linear gradient (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 8.0 with a linear 10-500 mM imidazole gradient) using an AKTA Purifier 10 (GE Healthcare). Buffer exchange of pooled fractions containing the purified protein was accomplished using a PD-10 column (GE Healthcare) eluted with 25 mM sodium phosphate buffer, pH 7.5. Final purified protein was drop frozen in liquid nitrogen and stored at -80 °C. Protein purity was confirmed by SDS-PAGE and protein concentration was determined using the Bradford Protein Assay Kit (from Bio-Rad, Hercules, CA, USA) using BSA as a standard. Small aliquots of protein were thawed for experiments and used fresh as required.

## 3. Supplementary Figures and Tables.

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```

**Figure S1.** Codon optimized sequence of AtmS14.

**Table S1.** Sugar $^{1}H$ and $^{13}C$ chemical shift values (in ppm) of **2-T**, **3-T** and **4-T**.

| Atom | 2-T | 3-T | 4-T |
|---|---|---|---|
| H1', C1' | 5.57, 97.2 | 5.60, 99.7 | 5.57, 98.5 |
| H2',C2' | 3.49, 74.0 | 3.56, 75.6 | 3.65, 73.28 |
| H3', C3' | 3.76, 75.3 | 3.76, 75.0 | 3.80, 75.0 |
| H4', C4' | 3.44, 71.5 | 3.49, 77.6 | NA |
| H5a', H5b', C5' | 3.87, 75.5 | 4.11, 77.5 | 3.91,3.55, 67.9 |
| H6a', H6b', C6' | 3.83, 3.77, 62.3 | NA | NA |

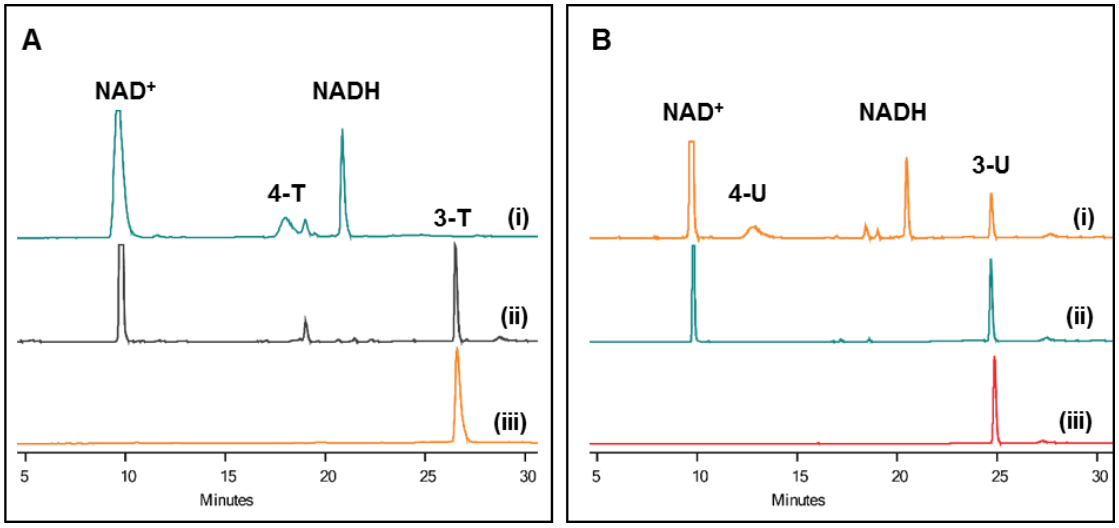



**Figure S2.** HPLC chromatograms of AtmS9-catalyzed reactions with **3-T (A)** and **3-U (B)** as substrates: **(i)** full reaction (2 mM **3-T** or **3-U**, 6 mM $NAD^+$, 15 μM AtmS9, 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C for 16 h); **(ii) 3-T** or **3-U** (2 mM) and $NAD^+$ (6 mM) standard mixture; and **(iii) 3-T** or **3-U** standard (2 mM).

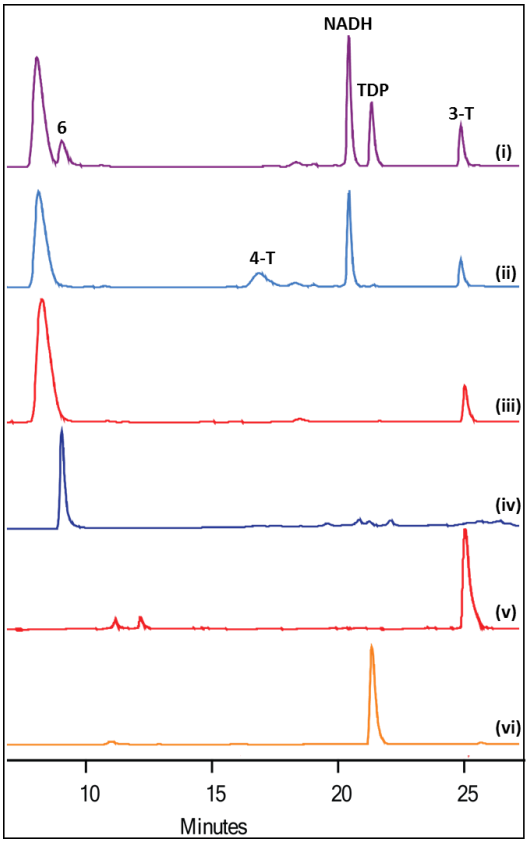



**Figure S3.** HPLC chromatograms the AtmS9/AtmS14-coupled reaction involving: (**i**) full reaction containing 2 mM **3-T**, 6 mM $NAD^+$, 15 µM AtmS9, 24 µM AtmS14 in 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C for 15 h; (**ii**) AtmS9 reaction in 2 mM **3-T**, 6 mM $NAD^+$, 15 µM AtmS9, 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C for 15 h; (**iii**) **3-T** (2 mM) and $NAD^+$ (6 mM) standard mixture; (**iv**) **6** standard (2 mM); (**v**) **3-T** standard (2 mM); and (**vi**) TDP standard (2 mM).

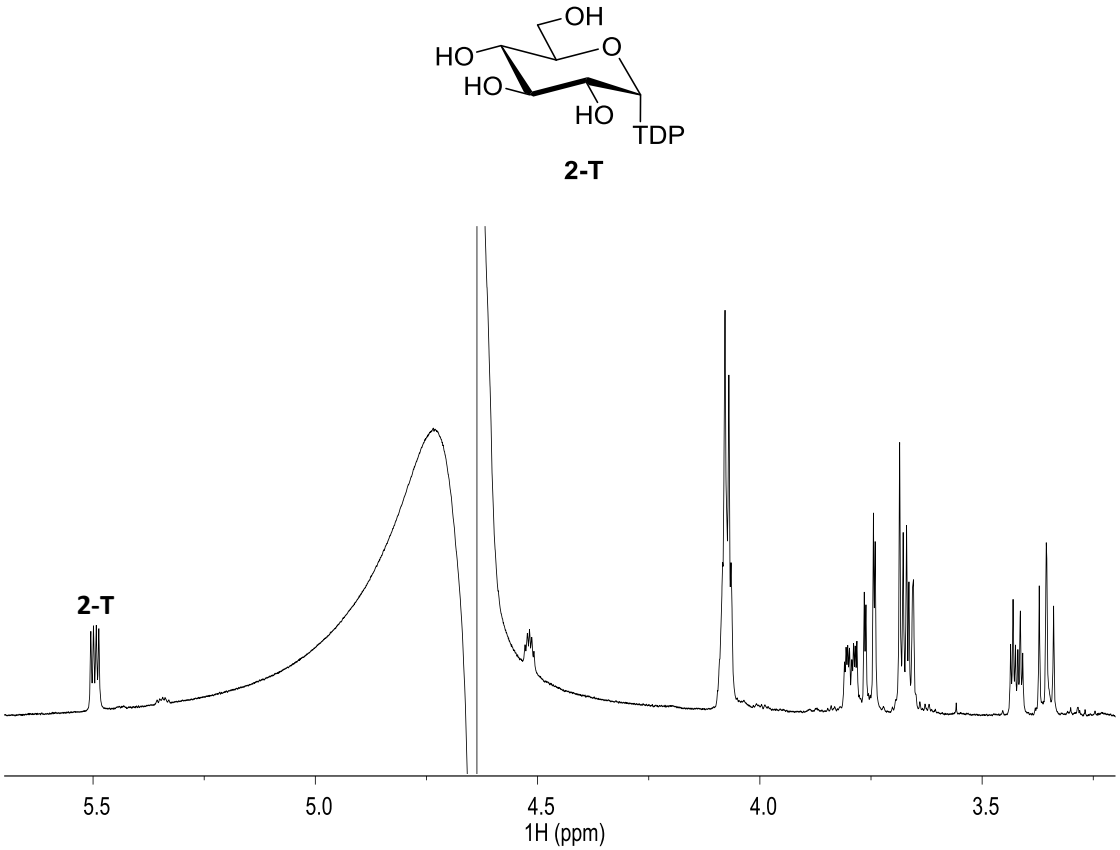



**Figure S4.** 1D-$^{1}$H NMR displaying key sugar resonances of a 2 mM **2-T** standard in 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C, where the **2-T** anomeric proton signal is labeled.

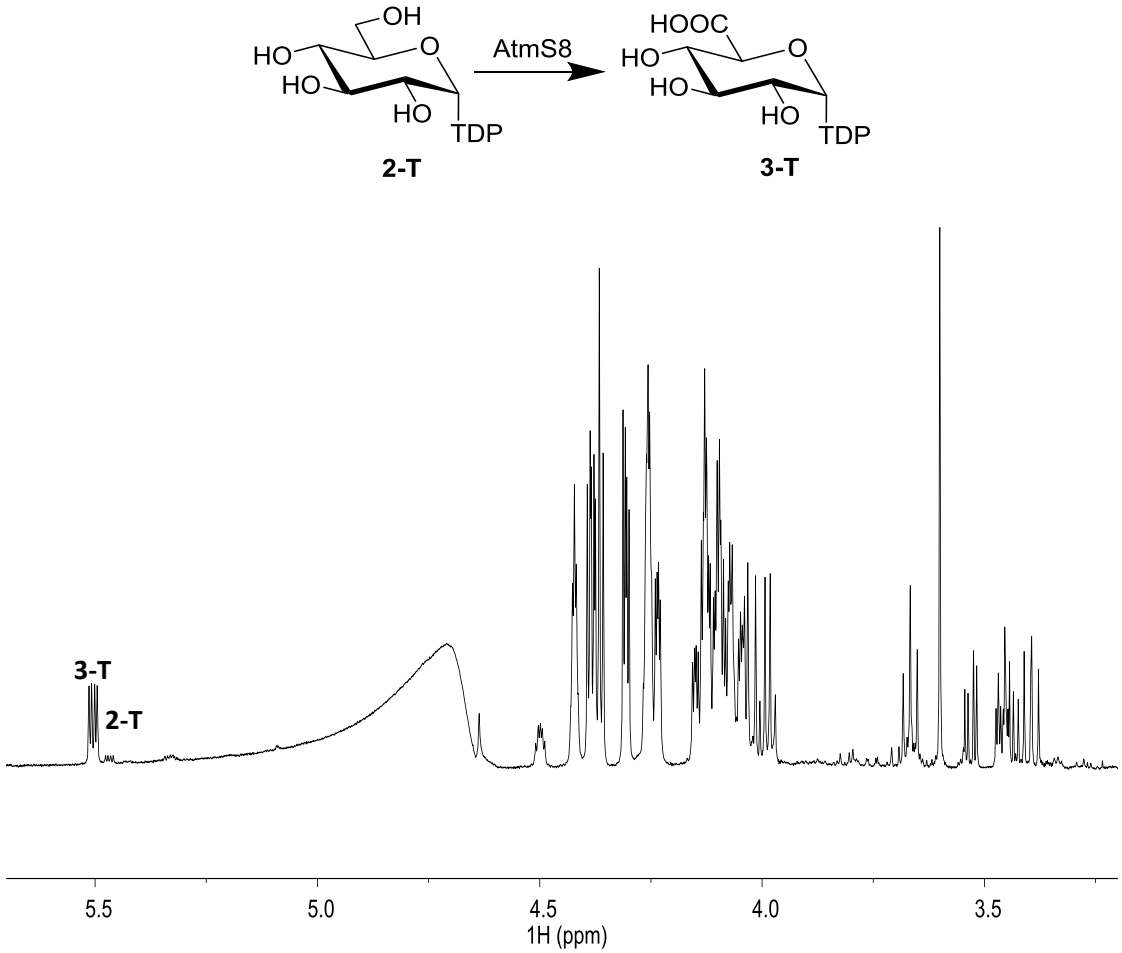



**Figure S5.** 1D-$^{1}$H NMR displaying key sugar resonances of a reaction containing 2 mM **2-T**, 6 mM $NAD^{+}$, 10 µM AtmS8 in 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C after 16 h where anomeric proton signals of **2-T** and **3-T** are labeled.

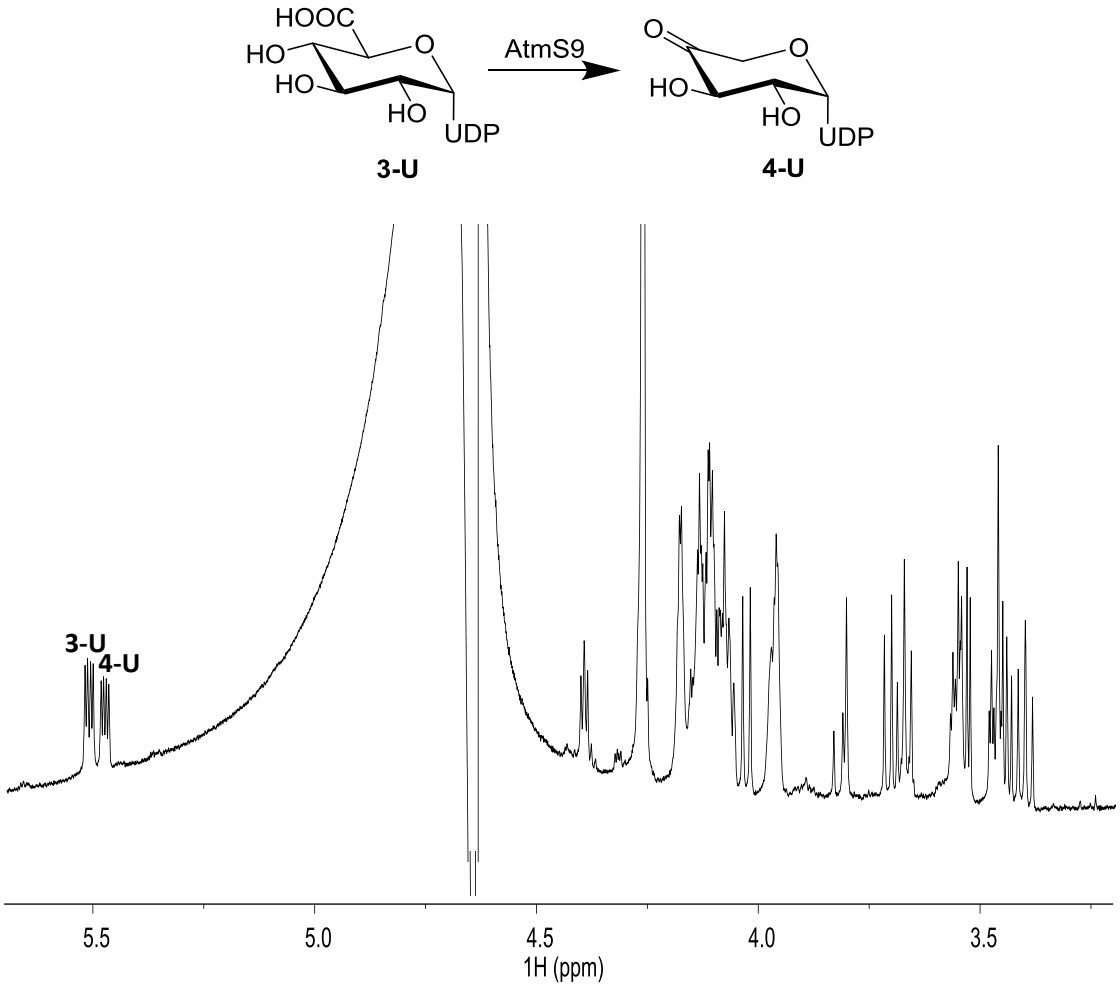



**Figure S6.** 1D-$^{1}$H NMR displaying key sugar resonances of an AtmS9-catalyzed reaction containing ~2 mM **3-U** from AtmS8 coupled reaction [2 mM **2-U**, 6 mM $NAD^{+}$, 10 µM AtmS8 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C for 20 h] and 15 µM AtmS9 after 4.5 h where the anomeric protons of **3-U** and **4-U** are labeled.

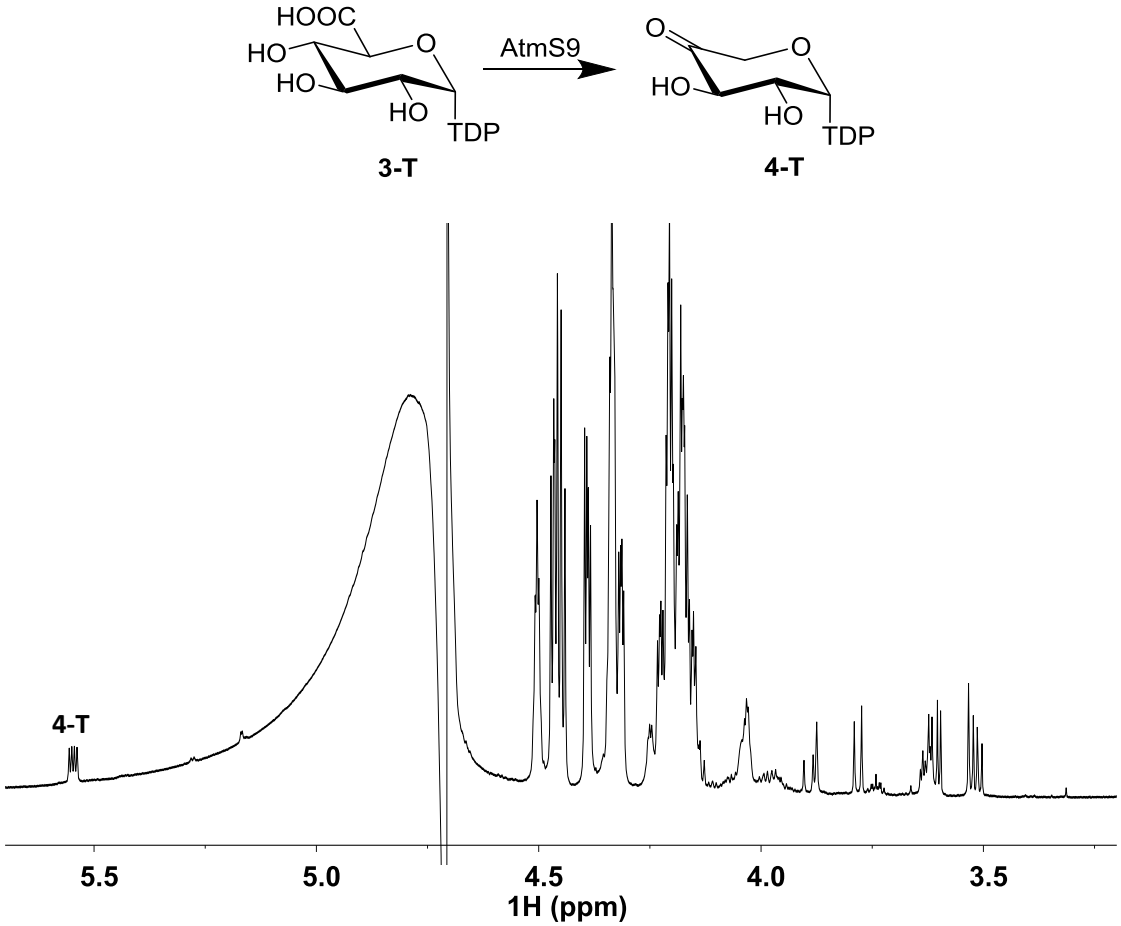



**Figure S7.** 1D-$^{1}$H NMR displaying key sugar resonances of an AtmS9-catalyzed reaction containing ~2 mM **3-T** from an AtmS8 coupled reaction [2 mM **2-T**, 6 mM $NAD^{+}$, 10 µM AtmS8 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C for 20 h] and15 µM AtmS9 after 15 h where the anomeric proton of **4-T** is labeled.

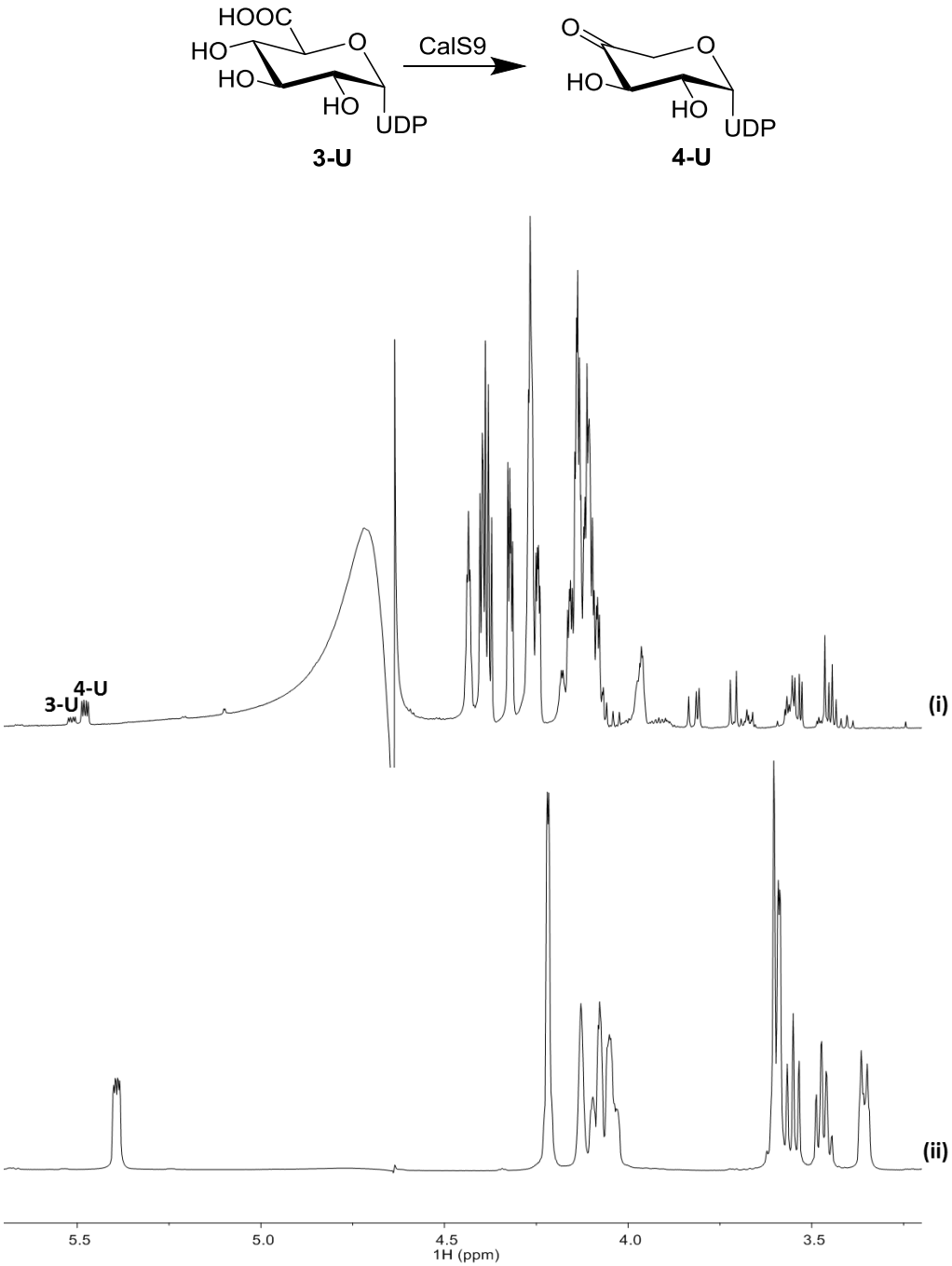



**Figure S8.** 1D-$^{1}$H NMR spectra displaying key sugar resonances of (**i**) a CalS9-catalyzed reaction containing 2 mM **3-U**, 6 mM $NAD^{+}$,15 µM CalS9, 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C and 12 h where the anomeric signals of **3-U** and **4-U** are labeled; and (**ii**) standard UDP-xylose.

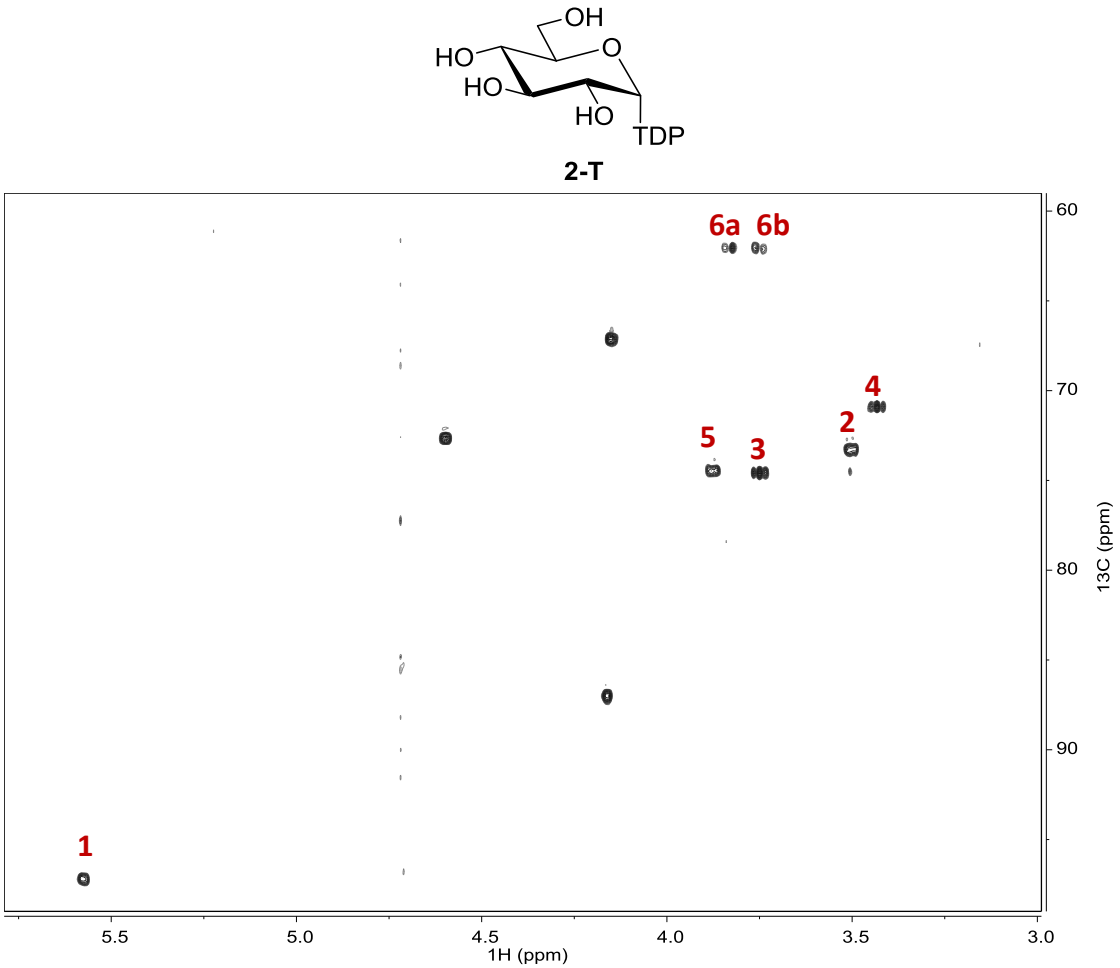



**Figure S9.** 2D-$^{1}$H-$^{13}$C HSQC spectra displaying key sugar resonances of a 2 mM **2-T** standard in 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C where the sugar resonances of **2-T** are labeled.

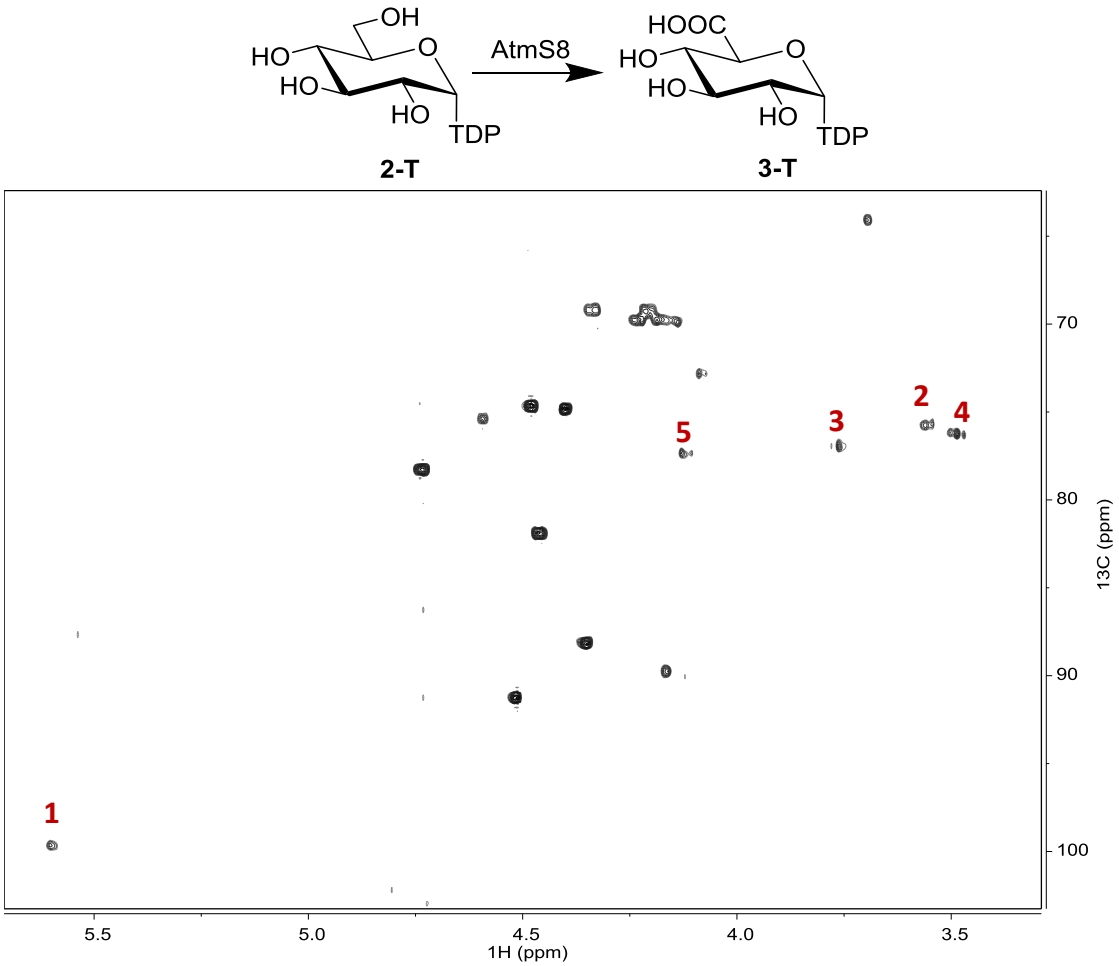



**Figure S10.** 2D-$^{1}$H-$^{13}$C HSQC spectra displaying key sugar $^{1}$H-$^{13}$C correlations of an AtmS8-catalyzed reaction containing 2 mM **2-T**, 6 mM $NAD^{+}$, 10 μM AtmS8 in 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C after 16 h where the sugar resonances of **3-T** are labeled.

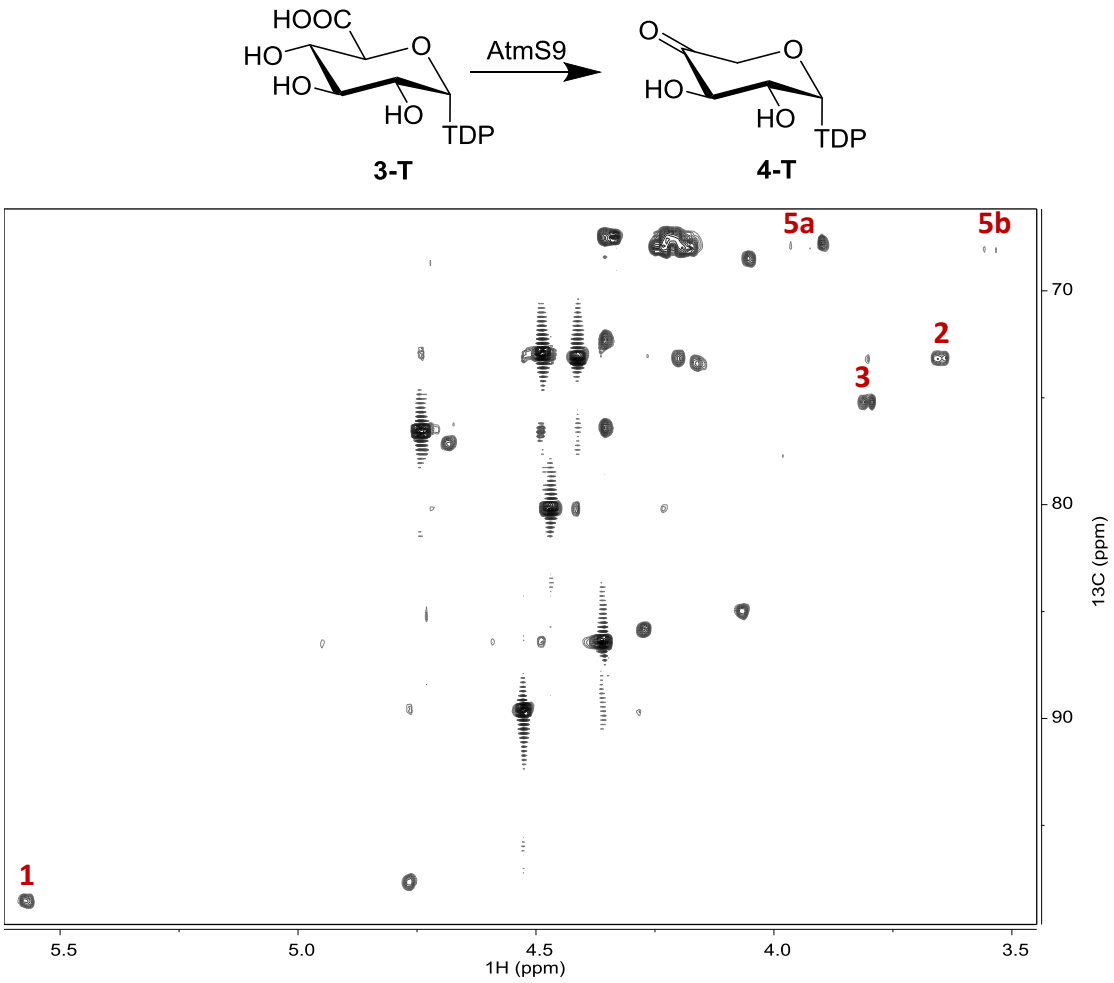
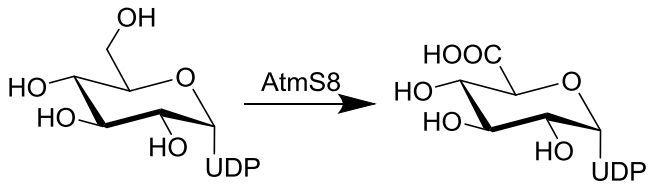



**Figure S11.** 2D-$^{1}$H-$^{13}$C HSQC spectra displaying key sugar resonances of an AtmS9-catalyzed reaction (containing ~70% $D_2O$) starting from ~2 mM **3-T** from an AtmS8-catalyzed coupled reaction [2 mM **2-T**, 6 mM $NAD^+$,10 µM AtmS8 in 1 mM $MgCl_2$, 1 mM DTT in 50 mM $NaH_2PO_4$ buffer, pH 7.5 at 30 °C for 16 h] and 15 µM AtmS9 after 15 h with the sugar resonances of **4-T** labeled. The weak intensities of 5a and 5b are attributed to deuterium incorporation from the solvent during the reaction.

**4. HRMS of AtmS8, AtmS9, CalS9 and AtmS14-catalyzed reactions**.




**2-U** **3-U**

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|---|---|
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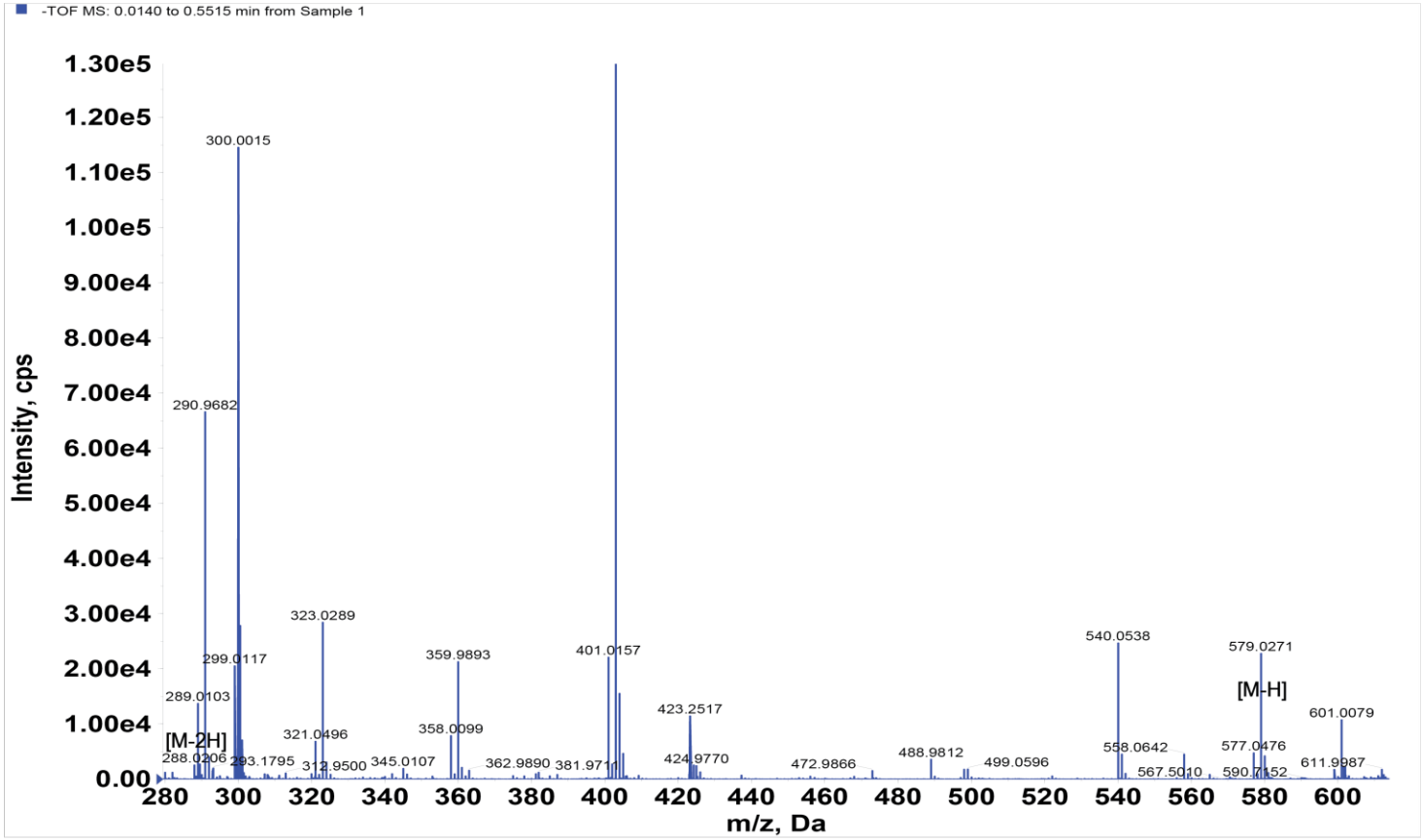

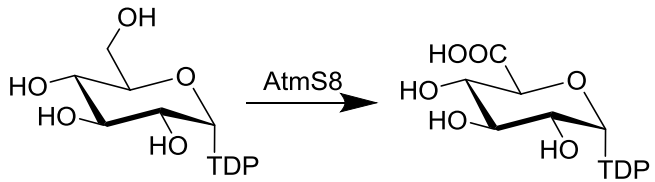


**2-T** **3-T**

| | |
|---|---|
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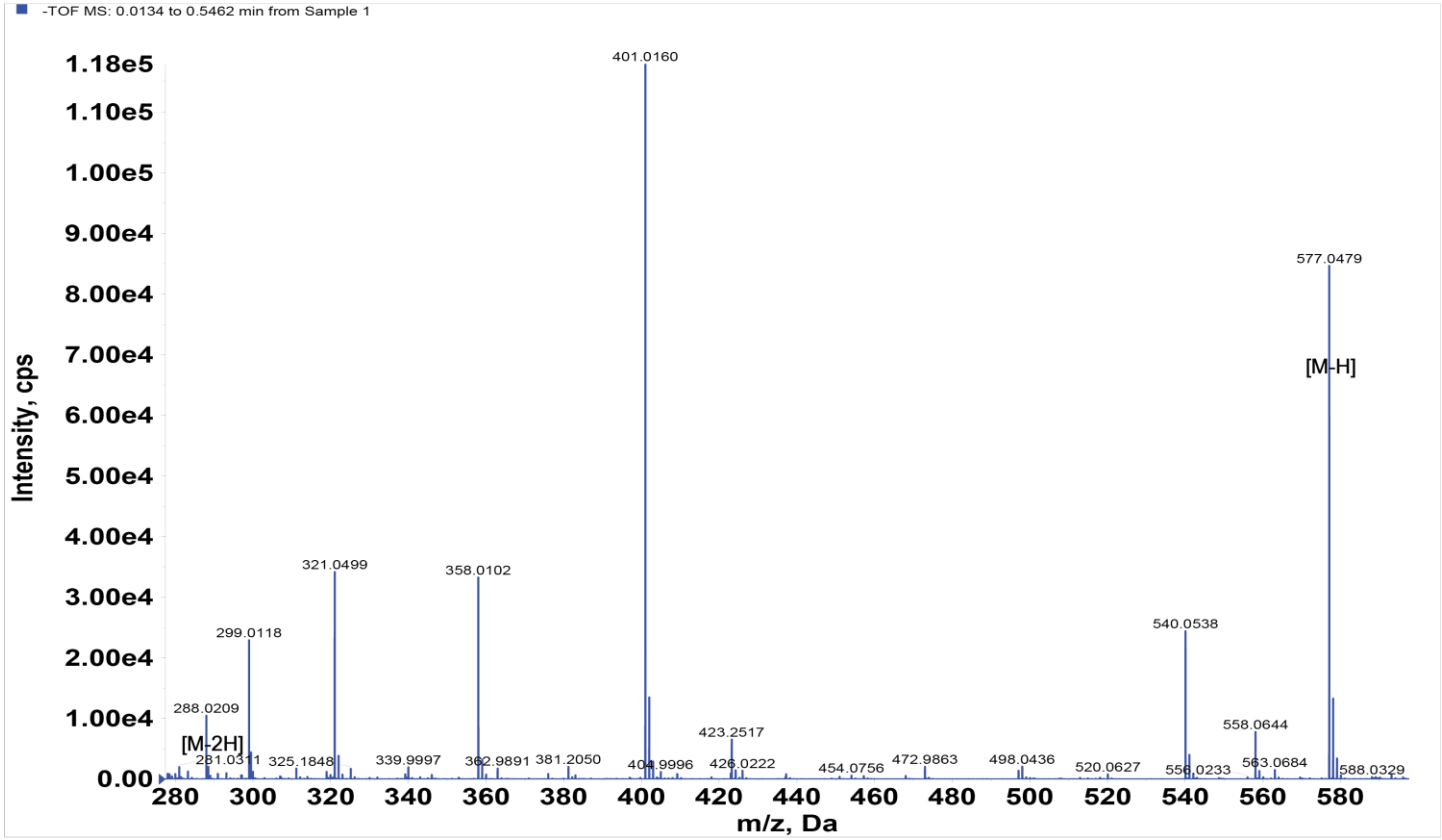

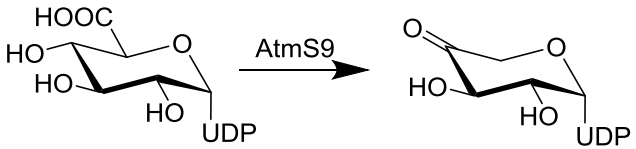


**3-U** **4-U**

| | |
|---|---|
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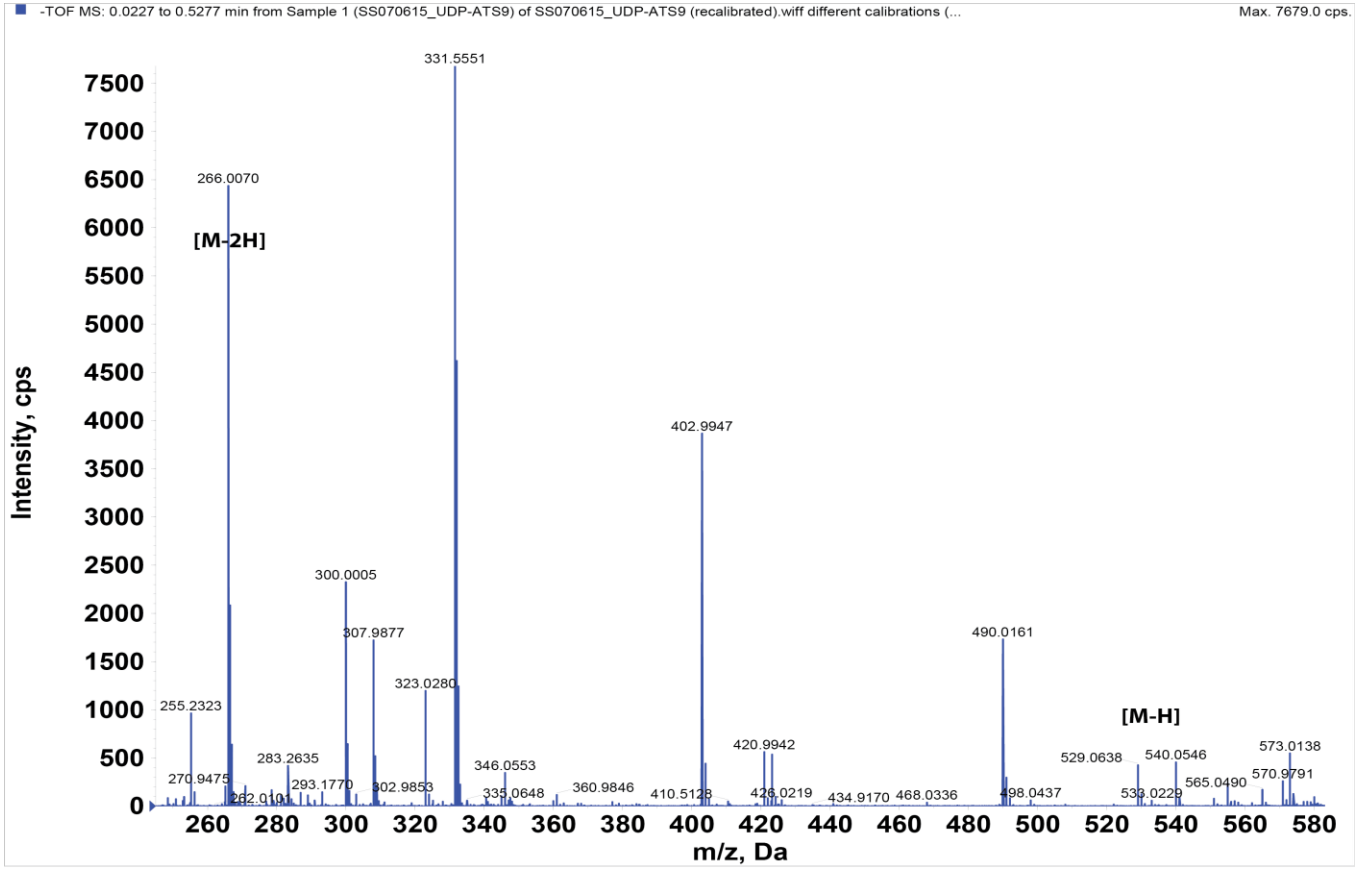

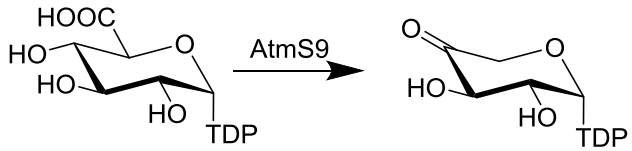


**3-T** **4-T**

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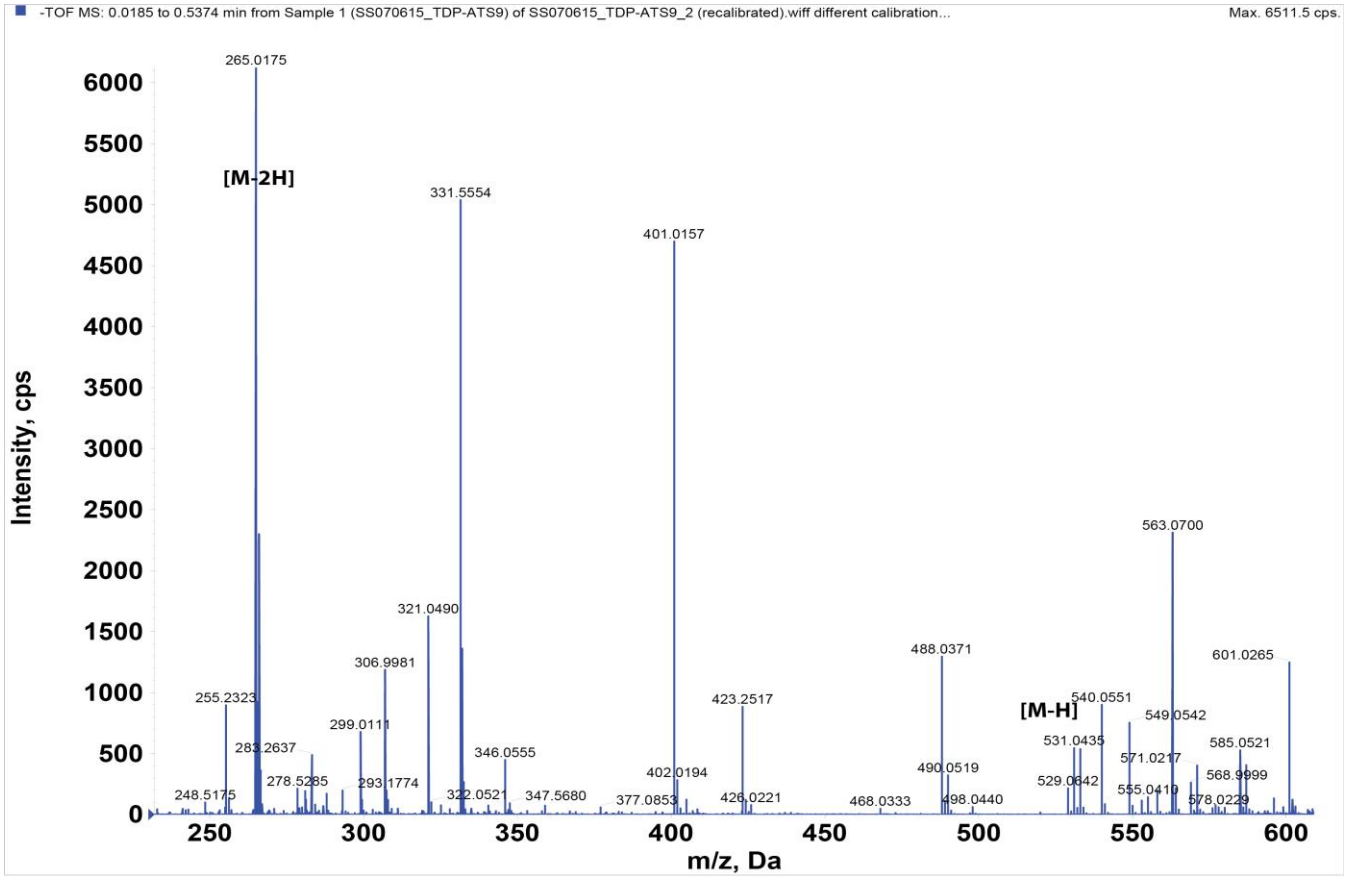

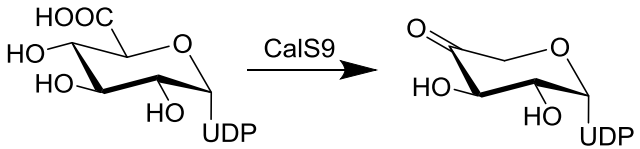


**3-U** **4-U**

| | |
|---|---|
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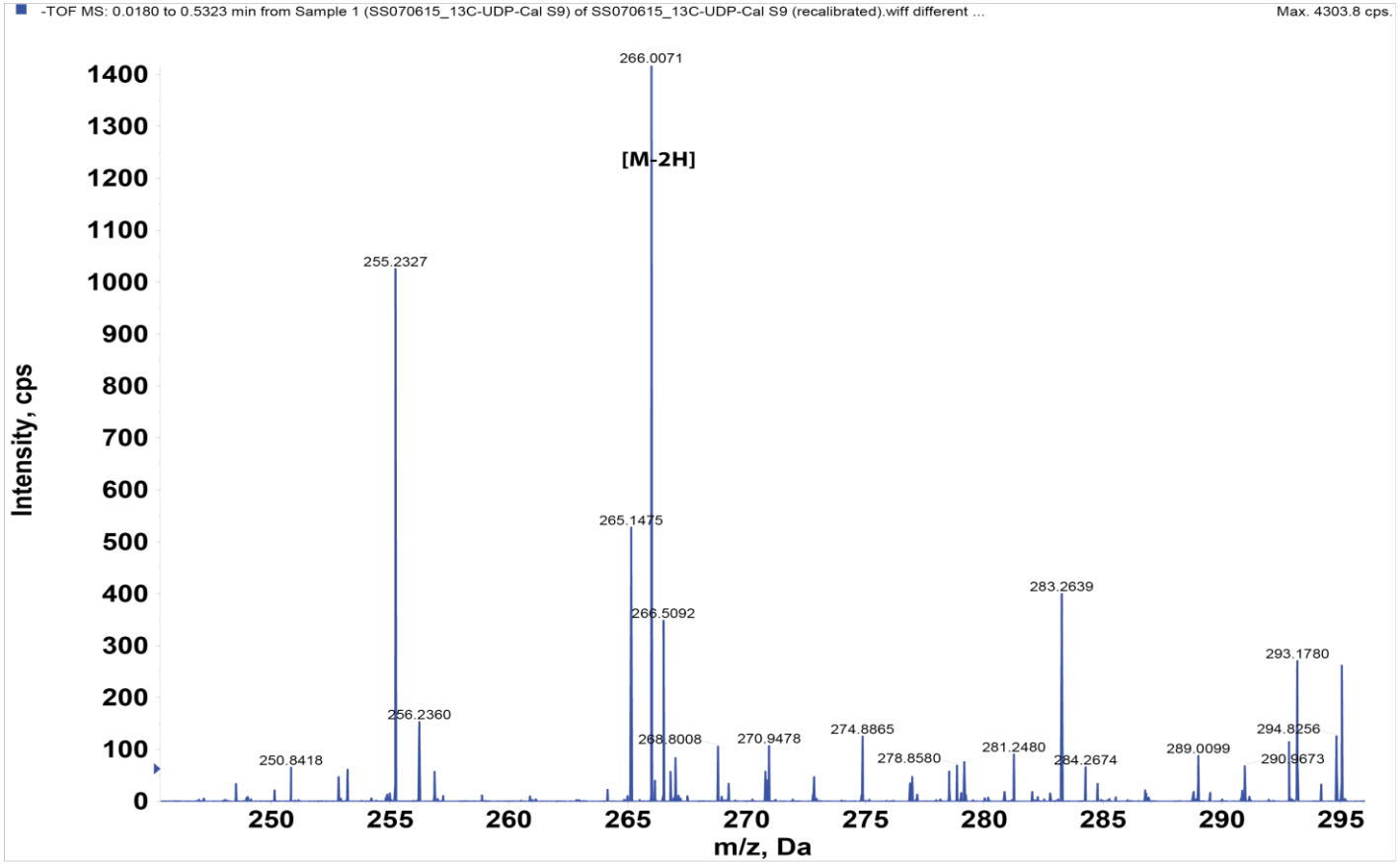

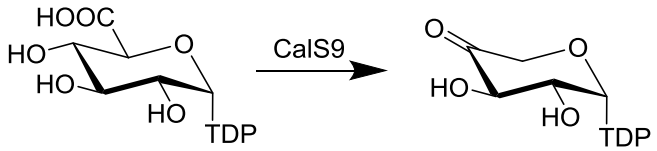


**3-T** **4-T**

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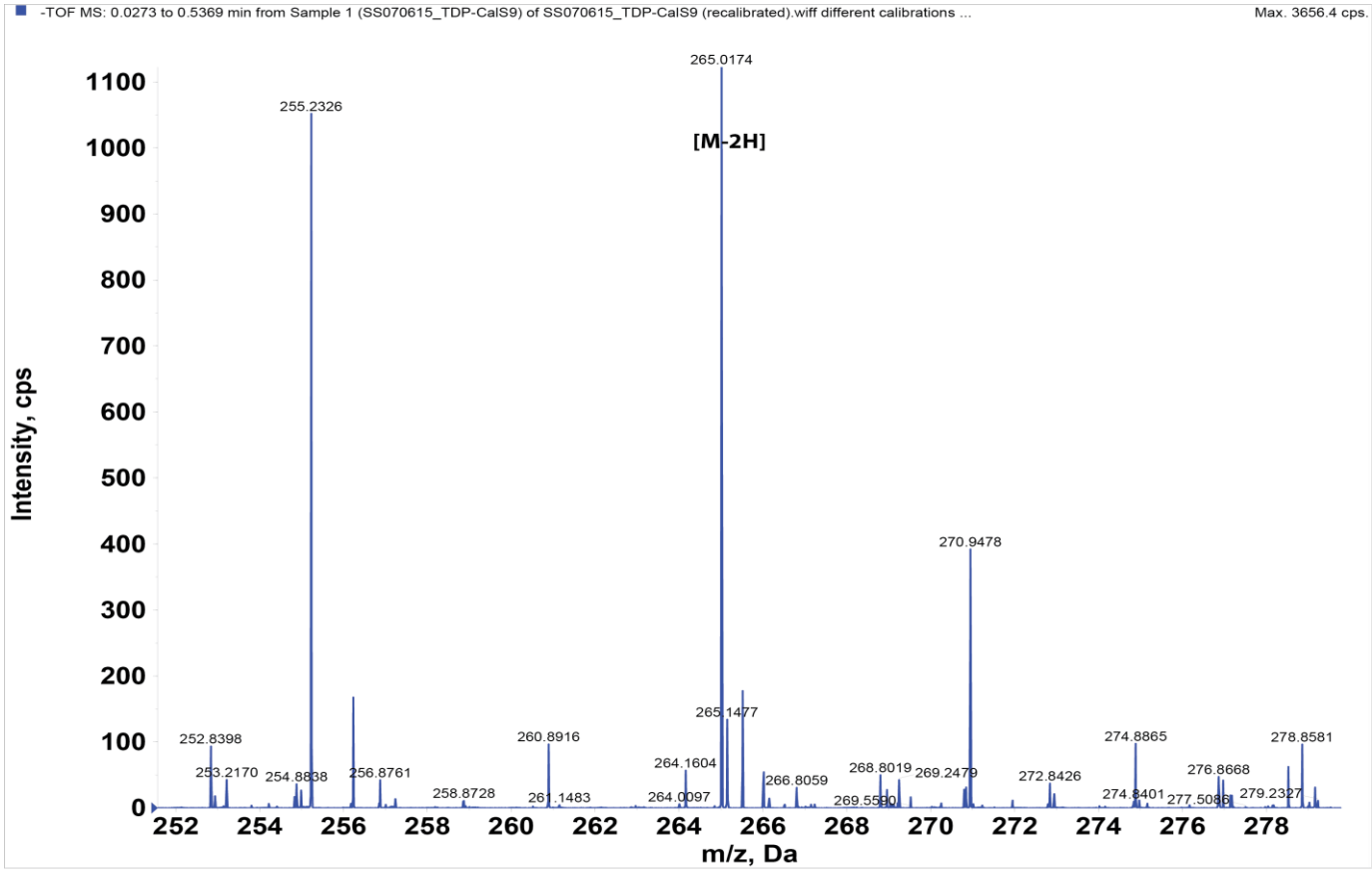

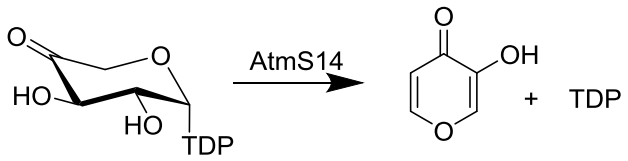


**4-T** **5**

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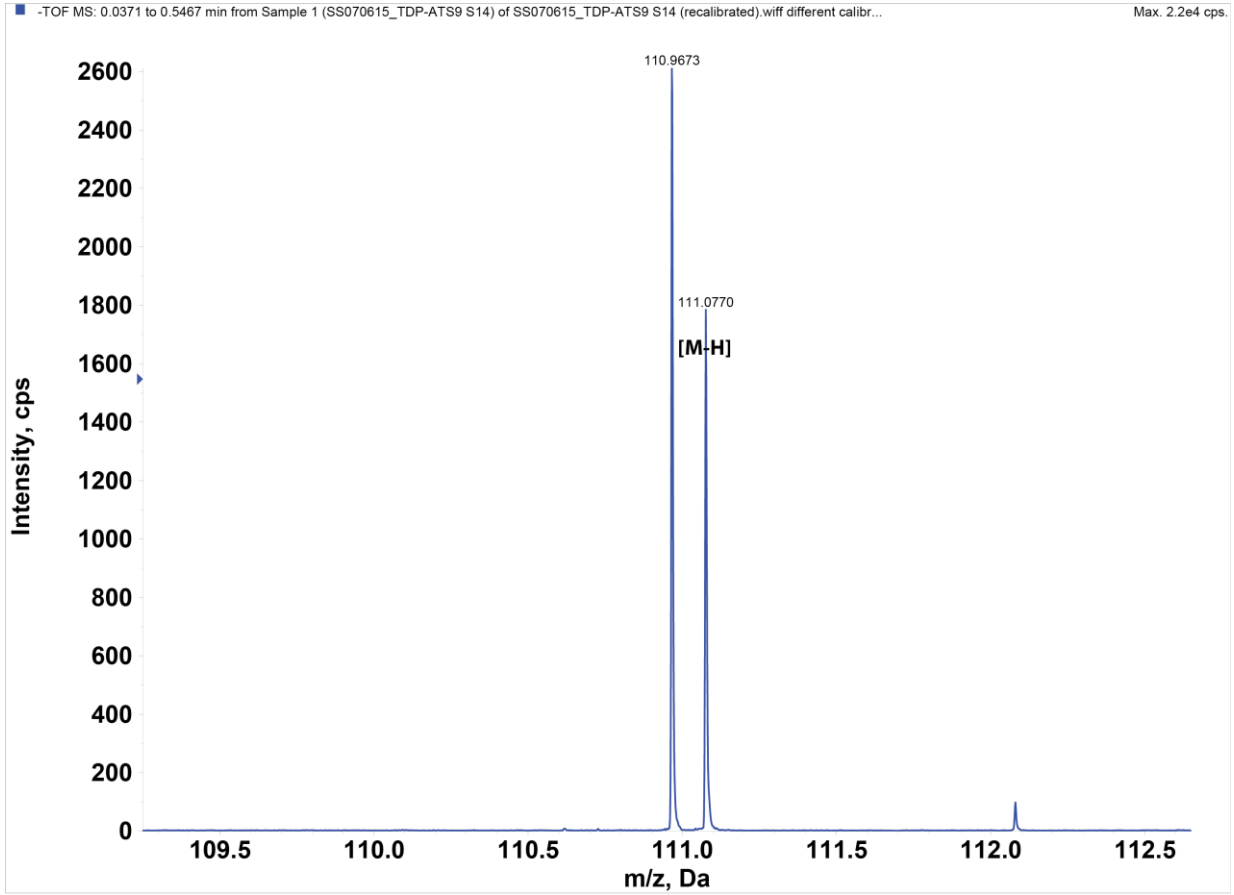
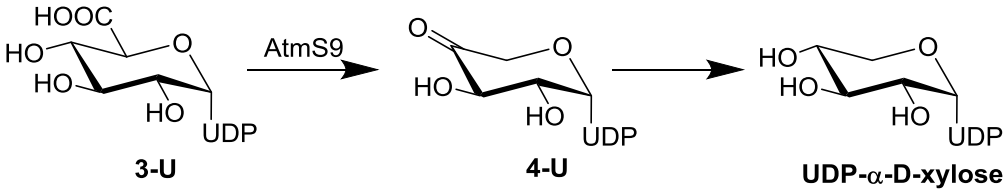
obs m/z ($[M-H]^{-1}$) 111.0770






**3-U** **4-U** **UDP-α-D-xylose**

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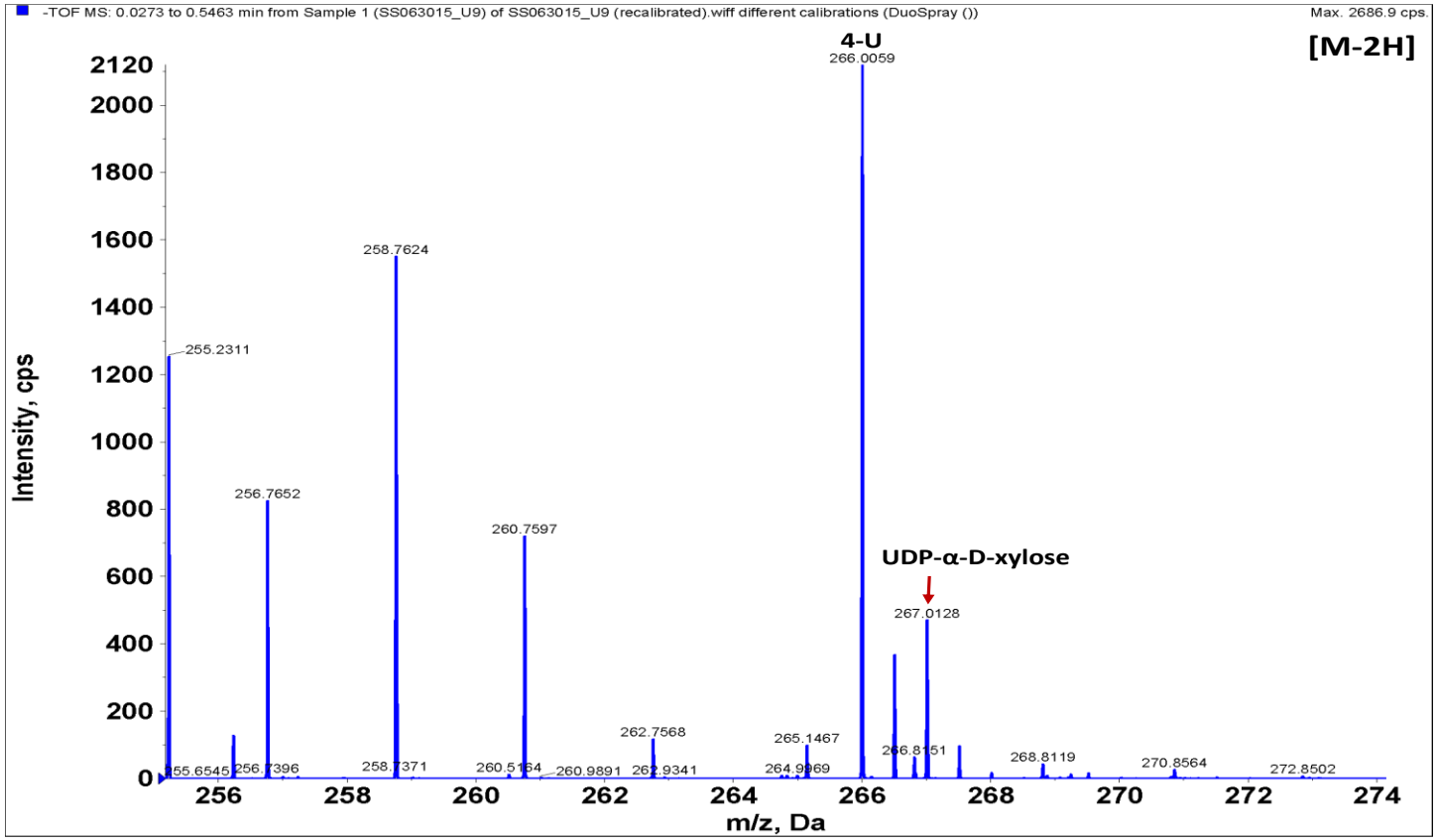

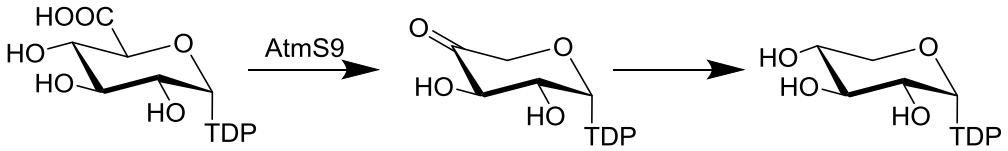


**3-T** **4-T** **TDP-α-D-xylose**

| | | |
|---|---|---|
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| obs m/z ($[M-2H]^{-2}$) | 265.0159 | 266.0237 |