A unifying nitrososynthase involved in nitrosugar biosynthesis

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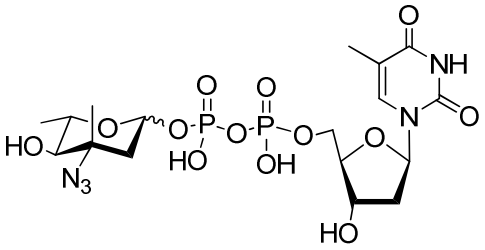
Supplementary Data

Bacterial strains, Plasmids and Materials. All reagents were obtained from Sigma-Aldrich corporation and used without further purification unless otherwise noted. *E. Coli* TOP10 and BL21(DE3) competent cells were obtained from Invitrogen Inc. (Carlsbad, CA) and Novagen (Madison, WI), respectively. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA). The pET28a expression vector was purchased from Novagen Inc. Taqplus DNA polymerase was purchased from Stratagene Inc. (La Jolla, CA). DNA primers were obtained from Operon Biotechnologies (Huntsville, AL). Solvents were reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on glass plates precoated with silica gel (250 µm, Sorbent Technologies), with detection by UV and/or spraying with H_2SO_4 (50%). Flash chromatography was carried out on silica gel (60 Å, 32-63 µm), purchased from Sorbent Technologies. Analytical HPLC of synthetic reaction mixtures was performed on a Hewlett-Packard 1100 series instrument using a Phenomenex Luna 5 µm C18 column (250 mm x 4.6 mm). Compounds bearing a thymidine chromophore were monitored at an absorbance of 270 nm. Synthetic reactions were monitored by HPLC using gradient A (a linear gradient from H_2O/0.1% NH_4HCO_3 to 100% MeOH/0.1% NH_4HCO_3 over the course of 25 min). Preparative HPLC was performed on a Varian ProStar instrument using a Phenomenex Luna 10 µm C18 column (250 mm x 50 mm). NMR spectra were recorded on Varian Innova 400 or 500 MHz spectrometers. Mass spectra (ESI) for synthetic compounds were obtained using an Agilent Technologies LC/MSD instrument (Model #G1956B).



Thymidine 5'-(3-amino-2,3,6-trideoxy-3-C-methyl-α,β-l-arabino-hexopyranosyl diphosphate), TDP α,β-epi-l-vancosamine azide (**5**). The protected TDP *epi*-vancosamine (reference: Oberthur, M.; Leimkuhler, C.; Kahne, D., *Org Lett* **2004,** 6, (17), 2873-76) (38 mg, 60 µmol) was dissolved in MeOH/H_2O/Et_3N (2:2:1, 4 mL) and stirred at room temperature for 16 h. Following evaporation, the residue was redissolved in MeOH/H_2O (2:1, 2 mL) and purified by reversed-phase HPLC. **2.25** (18 mg, 92%) was obtained as its ammonium salt, t_R = 9.0 min, method A. ^{1}H NMR (400 MHz, D_2O): **2.25*β***: δ = 7.62 (s, 1 H, 6-H), 6.24 – 6.21 (m, 1 H, 1'-H), 5.22 – 5.17 (dt, $J_{1,2b} = J_{1,P}$ = 9.0, $J_{1,2a}$ = 2.1 Hz, 1 H, 1"-H), 4.47 (m, 1 H, 3'-H), 4.02 (m, 3 H, 4'-H, 5'-H_2), 3.52–3.47 (m, 1 H, 5"-H), 3.16 (d, $J_{4,5}$ = 10 Hz, 1 H, 4"-H), 2.22 - 2.08 (m, 2 H, 2'-H_2) 2.07 (d, ,$J_{2a,2b}$ = 12.6 Hz, 1 H, 2"-H_a), 1.71 (s, 3 H, thymidine CH_3), 1.56-1.51 (dd, $J_{1,2b}$ = 9.0, $J_{2a,2b}$ = 12.6 Hz, 1 H, 2"-H_b), 1.25 (s, 3 H, 3"-CH_3), 1.13 (d, $J_{5,6}$ = 6.4 Hz, 3 H, 6"-H_3); ^{31}P NMR (162 MHz, CD_3OD): δ = –10.61, –12.82. LRMS (ESI) for $C_{17}H_{27}N_5O_{13}P_2$ (570.12): 571 $[M–1H]^-$.

Cloning and overexpression of *ORF36*. The gene encoding ORF36 was amplified from genomic DNA of *Micromonospora carbonacea* var. *africana* (NRRL 15099) using the following primers: 5' GCACATATGGCGGCGGATCTTCGCGC3' and 5'TTGAAGCTTTTATTACGCCGAGGTCCGGGAGC 3' (*Nde*I and *Hind*III restriction enzyme sites underlined). PCR reactions were carried out using Taqplus DNA polymerase according to the manufacture's protocol. Subcloning of *orf36* into the *Nde*I/*Hind*III sites of pET28a yielded recombinant plasmid pET28-36N for expression as an *N*-terminal hexahistidine fusion protein with ORF36. Plasmid pET28-36N was transformed into *E. Coli* BL21(DE3) for heterologous expression of ORF36. Cultures of *E. Coli* BL21(DE3)/pET28-36N were grown at 37°C to an OD_{600} of 0.6, at which point the culture was induced with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and grown an additional 6 hours at 28°C. Cells were harvested by centrifugation and stored at -80°C until needed. IPTG induced *E. Coli* BL21(DE3)/pET28-36N cells were resuspended in buffer A (20mM Imidazole, 0.5 M NaCl, 20mM Tris-HCl, pH 7.5) and lysed by sonication. The lysate was loaded onto a charged 5-ml Histrap crude column (Amersham Biosciences) and purified by FPLC at a flow rate of 5 ml/min. The column was washed with buffer A (20mM Imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5) and buffer B (500mM Imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5) using a step gradient. Fractions containing ORF36 were analyzed by SDS-page and buffer was exchanged via a desalting column (HisTrap) using buffer C (20mM Tris-Cl, 1mM dithiothreitol and 5% glycerol, pH 7.5) and stored at -80 °C until assayed.

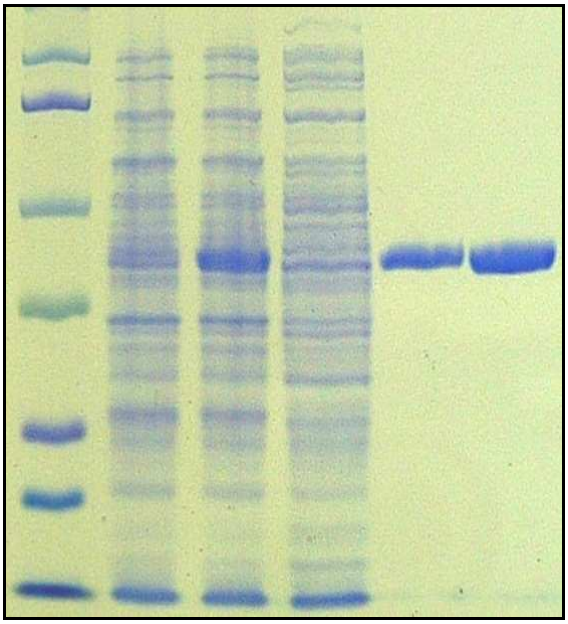


Figure S1. Overproduction of RubN8. From left to right, Lane 1: Molecular weight markers; Lane 2: Uninduced total protein extract; Lane 3 Induced total protein; Lane 4: Flow-through of Ni^{2+} column; Lane 5: Pooled fractions from Ni^{2+} column; Lane 6: Desalted fraction.

Cloning and overexpression of *RubN8*. The gene encoding RubN8 was amplified from *Streptomyces achromogenese* var. *rubradiris* (NRRL3061) genomic DNA. The following PCR primer pairs were used to clone the gene into the *NdeI/HindIII* site of pET28a (restriction sites underlined): 5'-TCCATATGA TGGAGACGGAACAGGCCCC-3 ' and 5'-TCAAGCTTTCACCGGCTGTCCACCGGC-3'. Restriction-digested PCR products were subcloned into the digested pET28a vector to yield pET28a-his$_6$-RubN8**.** Cultures of *E.coli* BL21(DE3) transformants in 1 L Luria broth (LB) media with 50 µg/mL kanamycin were grown at 37 ℃ to an OD_{600} of 0.6 at which point the cultures were induced with 0.25 mM isopropyl-1-thio-β-D-galactoside (IPTG) and grown for additional 16 h. Cells were harvested by centrifugation and frozen at -80℃ until needed. Frozen cells were resuspended in binding buffer [20 mM imidazole, 0.5 M NaCl, 20 mM sodium phosphate (pH 7.5)] and lysed by sonication. After clarification by centrifugation at 10000g, the lysates were loaded onto Ni^{2+}-charged 5-mL HisTrap column and purified using FPLC (Amersham Biosciences) with a flow rate of 5 mL/min. The column was washed with 50 mL of binding buffer and proteins were eluted with a gradient of imidazole (20-500 mM imidazole). Fractions containing the proteins were analyzed by SDS-PAGE, pooled, concentrated to 5 mL and desalted by FPLC using a 5 mL size exclusion desalting column (HiTrap) from Amersham Biosciences. The column was equilibrated with 50 mL desalting buffer [20 mM Tris.HCl, 5%glycerol, 1 mM DTT, pH 7.5] and the proteins were then loaded onto the column (maximum of 1.5 mL) and eluted at a flow rate of 5 mL/min. Fractions containing the protein were analyzed again by SDS-PAGE and stored at -80 ℃ until assayed.

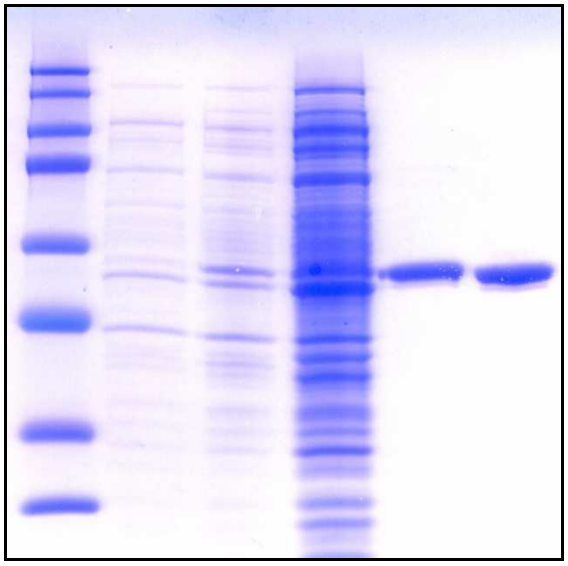


Figure S2. Overproduction of RubN8. From left to right, Lane 1: Molecular weight markers; Lane 2: Uninduced cell-free extract; Lane 3 Induced cell-free extract; Lane 4: Flow-through of Ni^{2+} column; Lane 5: Pooled fractions from Ni^{2+} column; Lane 6: Desalted fraction.

Preparation of L-TDP-*epi*-vancosamine (4). L-TDP-*epi*-vancosamine (4-*O*-desmethyl L-TDP-evernosamine) was prepared by reduction of a synthetic azide congener **5** (0.5 mM) with 1 mM tris(2-carboxyethyl)phosphine (TCEP) in 20 mM Tris-HCl (pH 7.5) at 22℃ for 24 hours. Amino sugar was stored in small aliquots at -80 ℃ until immediately prior to assays. HPLC/MS, performed for the reduction of the azide congener as described below for L-TDP-*epi*-vancosamine enzymatic reaction, indicated the extent of reaction was > 96%.

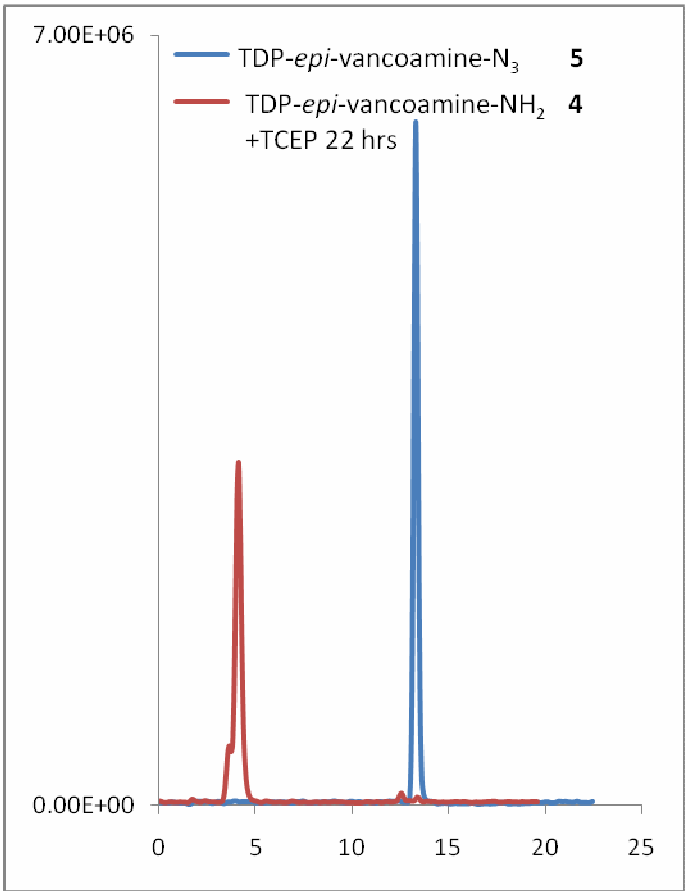


Figure S3. HPLC/MS analysis of the reduction of azide **5** to L-TDP-*epi*-vancosamine **4** (4-*O*-desmethyl L-TDP-evernosamine**.**

ORF36/RubN8 enzymatic reactions. Solutions of flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) were made in 20 mM Tris-HCl pH 7.5 immediately before use. The concentration of substrate L-TDP-*epi*-vancosamine **4** was determined by comparison of absorbance values of stock solutions to a TDP standard curve at 270 nm using a Nanodrop 1000 UV spectrometer (Thermo Fisher Inc). RubN8 and ORF36 were aliquoted in storage buffer [20 mM Tris.HCl, 5% glycerol, 1 mM DTT, pH 7.5], stored at -80 ℃ and thawed directly before use. Flavin reductase from *Photobacterium fischeri* (Roche Diagnostics GmbH, Mannheim, Germany) was stored in 40% glycerol, 1 mM EDTA, 0.1 mM DTT and 50 mM potassium phosphate, pH 7.0. The catalase and superoxide dismutase enzymes (Sigma Chemicals) were made in [20 mM Tris.HCl 40% glycerol, pH 7.5]. In a total volume of 50 µL, 128 - 250 µM of the substrate, L-TDP-*epi*-vancosamine, was incubated with 30 µM FAD, 1 U/mL catalase, 1 U/mL superoxide dismutase, 0.001 mg/mL flavin reductase, and 0.4 – 2.0 mM NADPH. Control assays lacking RubN8, FAD or NADPH were also performed. The reaction was initiated by the addition of RubN8 or ORF36. The time course of the reaction was followed by HPLC/MS at 30 ℃.

HPLC/MS assay for L-TDP-*epi*-vancosamine reaction. The products of oxidation of L-TDP-*epi*-vancosamine **4** to corresponding products were analyzed using Agilent 1100 HPLC system (Agilent, Palo Alto, CA), comprising a binary pump and refrigerated autosampler. Mass spectrometry was performed using a ThermoFinnigan (San Jose, CA) LCQ Quantum Deca XP ion trap mass spectrometer equipped with an API electrospray ionization source outfitted with a 50 µm I.D. deactivated fused Silica capillary. The injection volume was 5 µl. Products were separated using a Thermo Hypercarb column (3 × 50 mm). Mobile phase were: (A) H_2O with 50mM ammonium acetate and 0.1% (v/v) diethylamine and (B) H_2O/acetonitrile (5:95) with 50mM ammonium acetate and 0.1% (v/v) diethylamine. Gradient conditions were as follows: 0-5 min, B = 15%; 5-15 min, linear gradient to 35% B; 15 to 16 min, linear gradient to 100%B; 16 to 21 min, B = 100%; 21 to 22 min, linear gradient to 15% B; 22-30 min, B = 15%. The flow rate was maintained at 0.3 ml/min. The mass spectrometer was operated in both the negative ion and fill scan profile modes and the electrospray needle was maintained at 3,400 V. The ion transfer tube was operated at -47.50 V and 275℃. The tube lens voltage was set to -46 V. The collision energy for all product ion scans was set at 30%. Full product and ion scans were set as follows: 544→155-548; 558→155-560,; 560→155-562; 544→155-548; 570→155-575. Data were acquired in profile mode. The following optimized parameters were used for the detection: N_2 sheath gas 46 psi; N_2 auxiliary gas 13 psi; spray voltage 3.4 kV.

Figure S4. HPLC/MS analysis of RubN8 catalyzed oxidation reactions with 250 μM L-TDP-*epi*-vancosamine **4** and 2 mM NADPH.

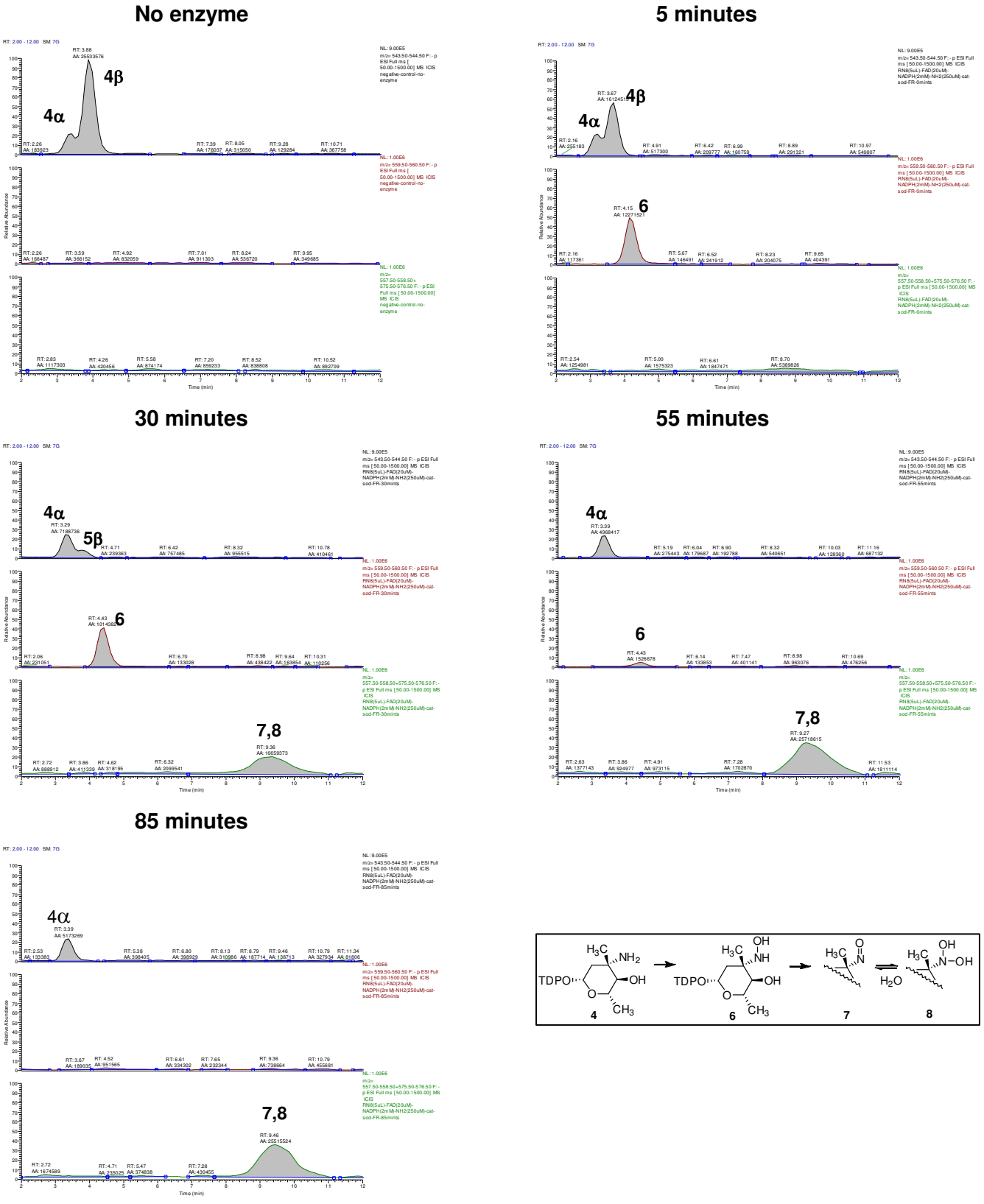
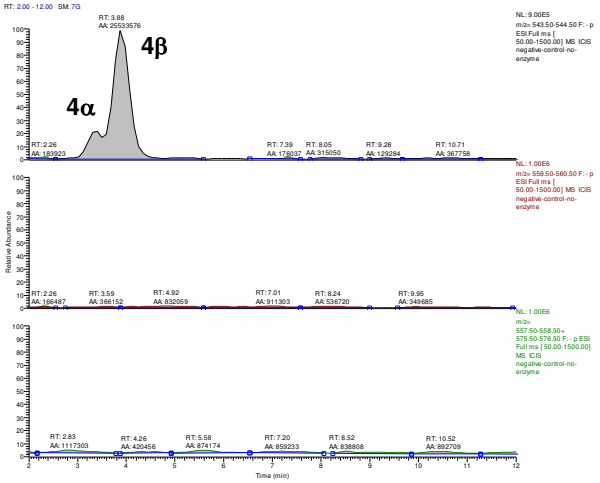
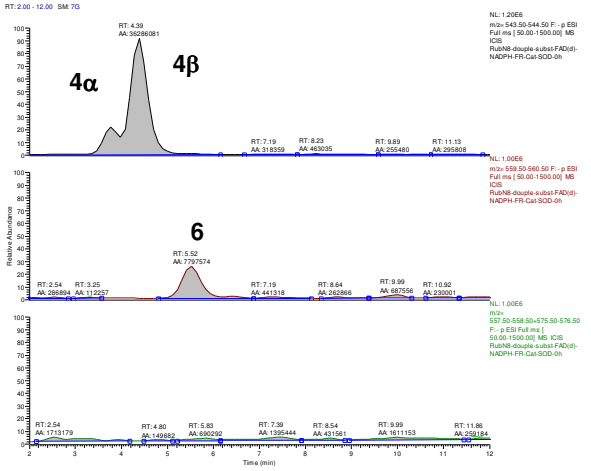


Figure S5. HPLC/MS analysis of RubN8 catalyzed oxidation reactions with 350 μM L-TDP-*epi*-vancosamine **4** and 400 μM NADPH.

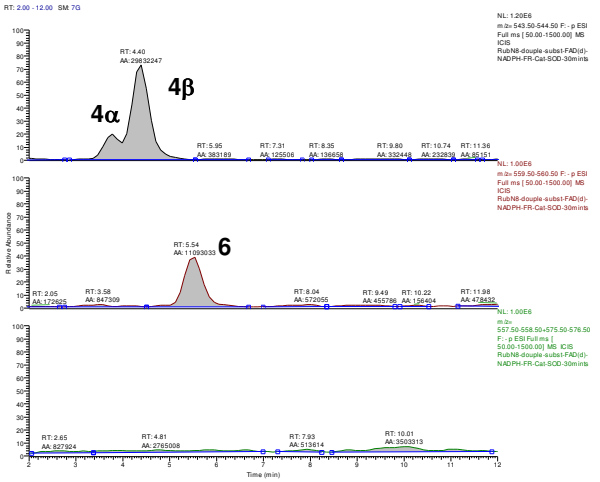
No enzyme



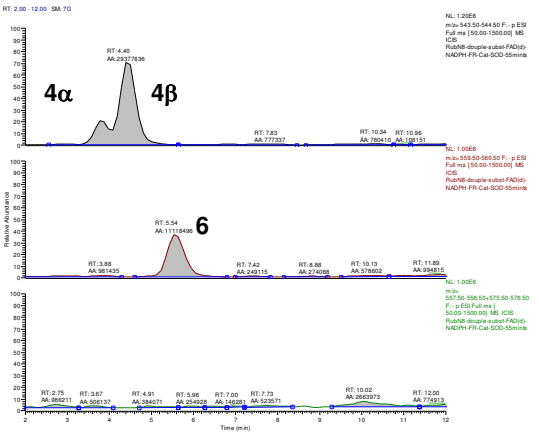
5 minutes



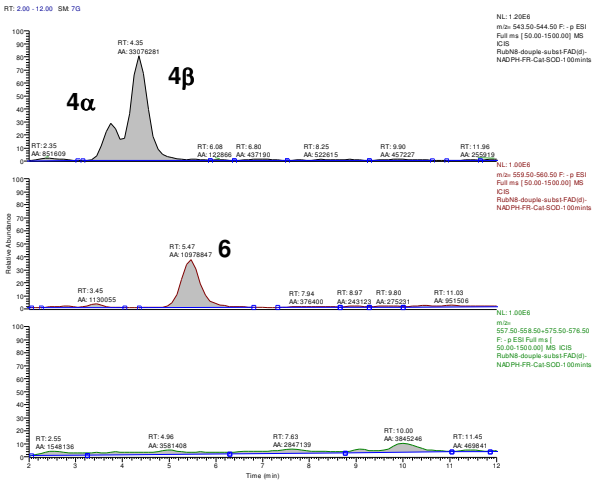
30 minutes



55 minutes



85 minutes



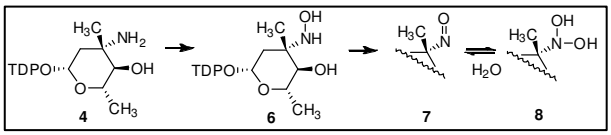
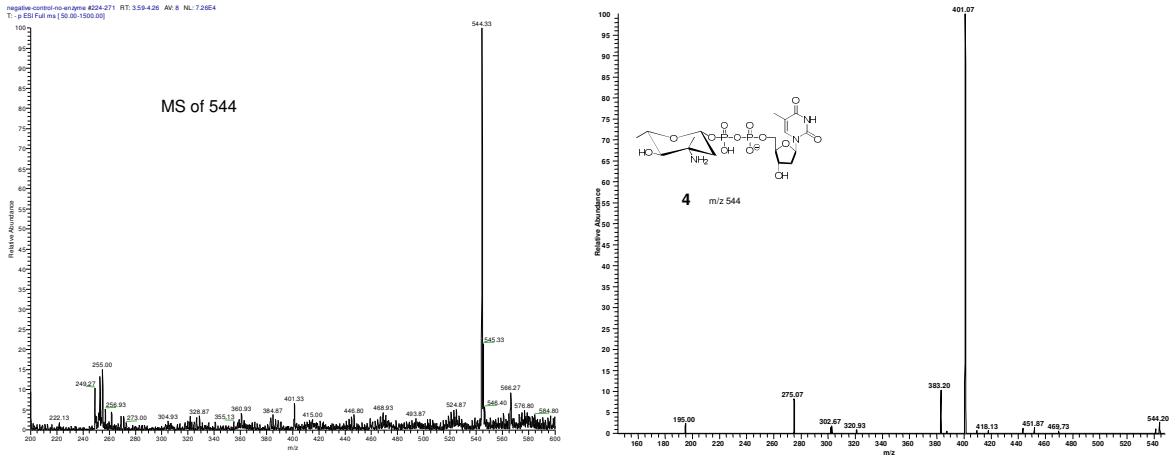
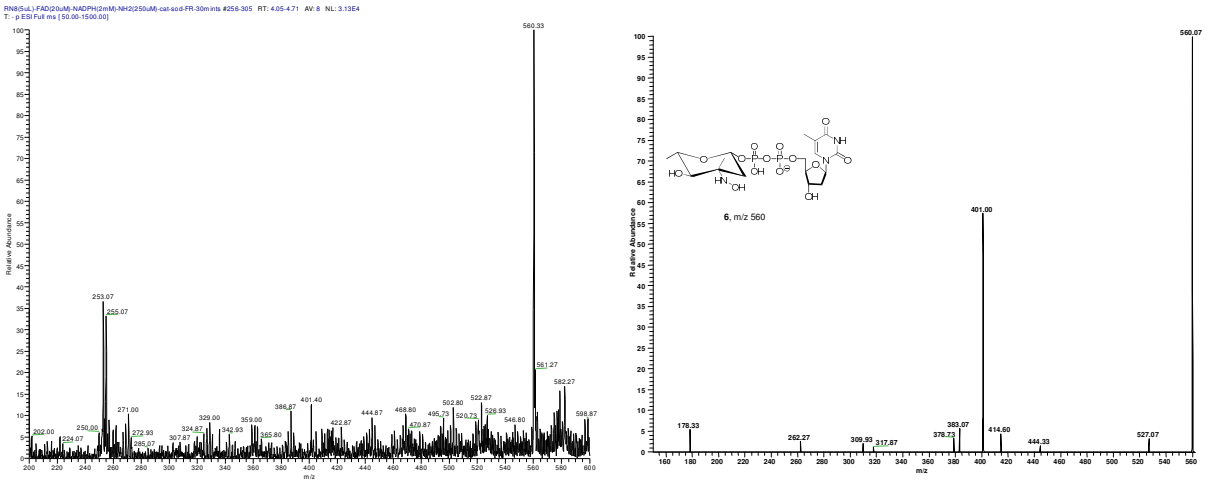


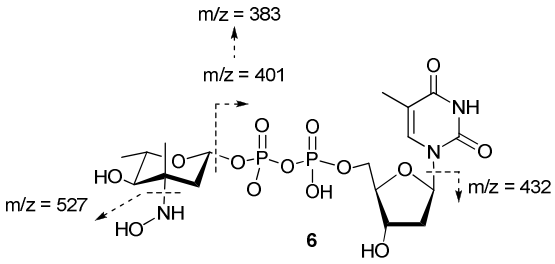
Figure S6. MS and Tandem MS of product ions for RubN8 catalyzed reactions

MS (left) and MS/MS (right) of L-TDP-*epi*-vancosamine **4**.



MS (left) and MS/MS (right) of L-TDP-epi-vancoshydroxylamine intermediate **6**

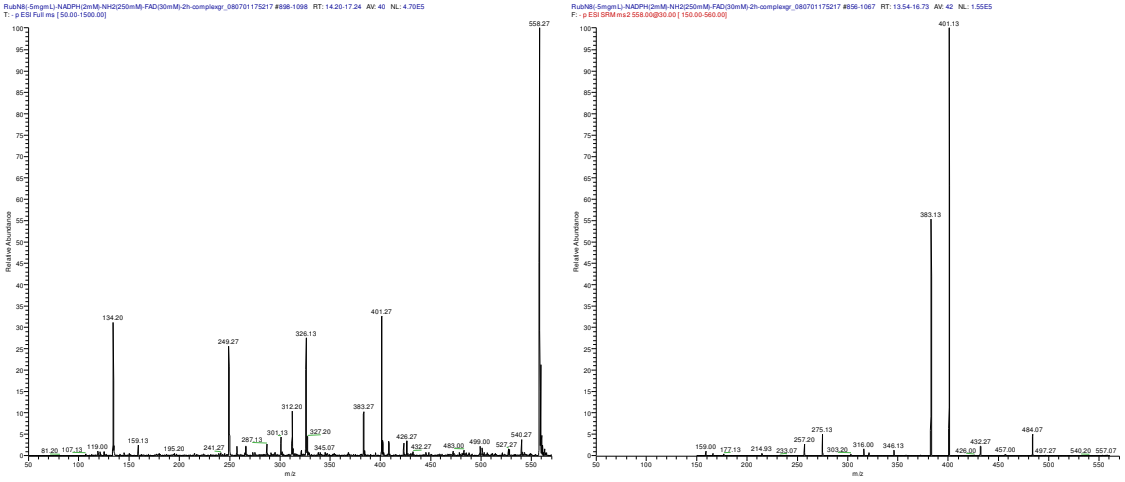




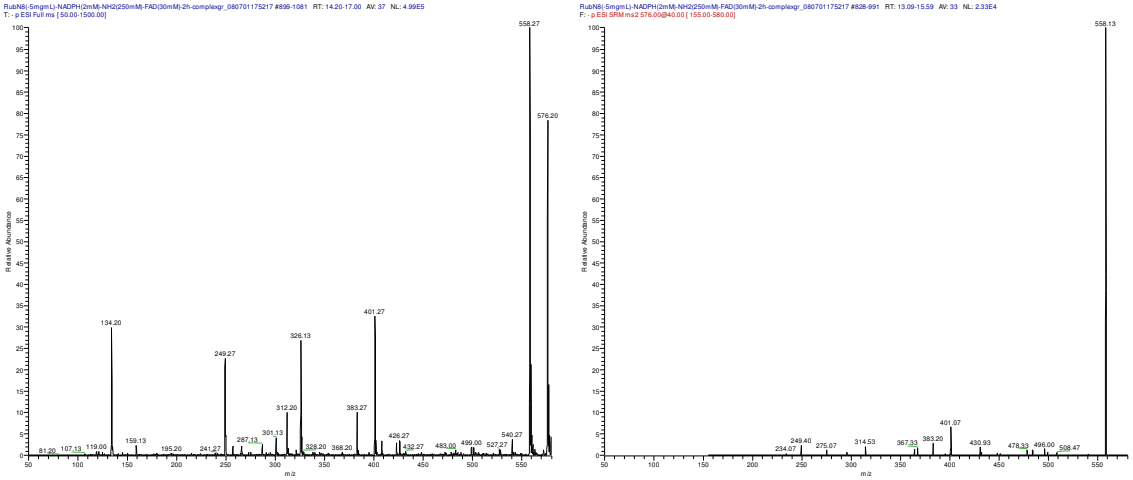
Chemical Formula: $C_{17}H_{28}N_3O_{14}P_2$
Exact Mass: 560.10465

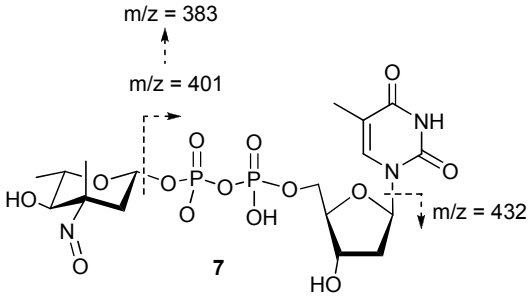
Figure S6 (con't). MS and Tandem MS of product ions for RubN8 catalyzed reactions

MS (left) and MS/MS (right) of L-TDP-*epi*-vanconitrose intermediate **7**.

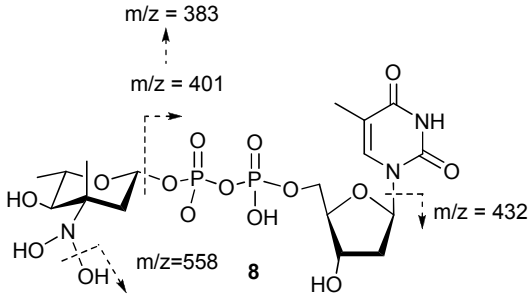


MS (left) and MS/MS (right) of L-TDP-epi-vanconitrose hydrate intermediate **8**





Chemical Formula: $C_{17}H_{26}N_3O_{14}P_2$
Exact Mass: 558.08900



Chemical Formula: $C_{17}H_{28}N_3O_{15}P_2$
Exact Mass: 576.09957

Figure S7. HPLC/MS analysis of ORF36 catalyzed oxidation reactions 120 uM **4** and 400 μM NADPH.

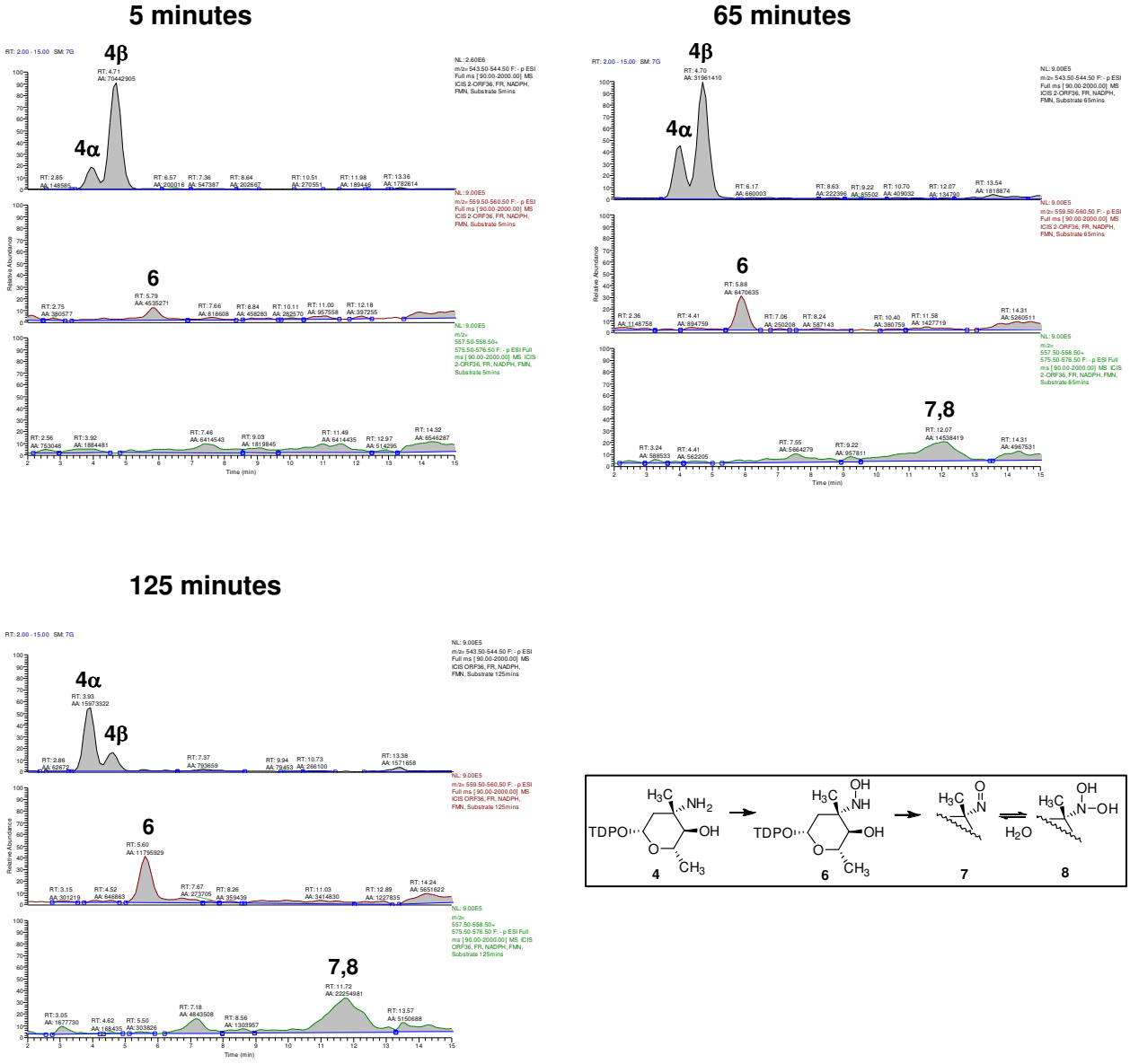
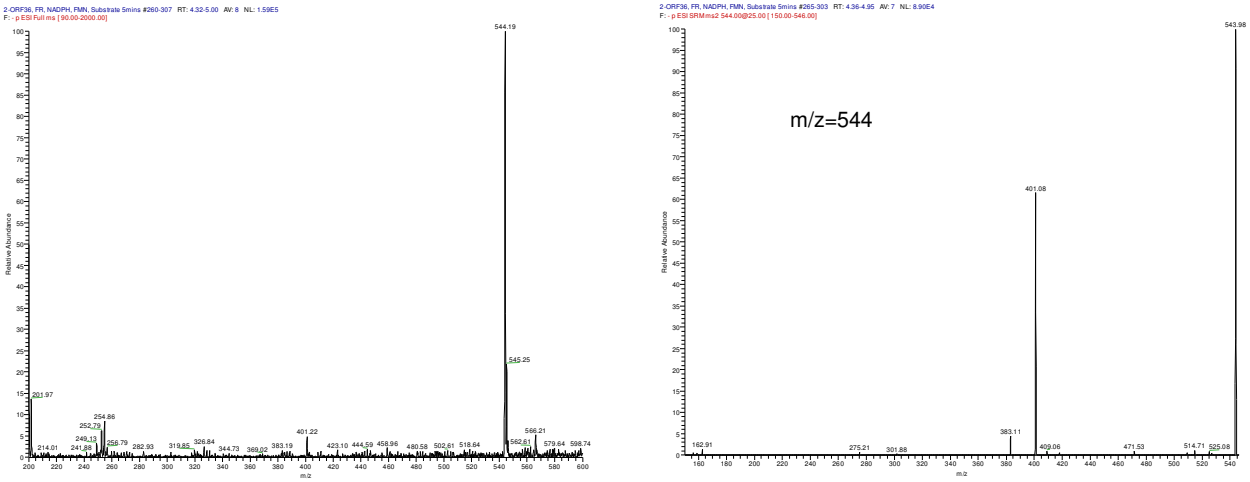


Figure S8. MS and Tandem MS of product ions for ORF36 catalyzed reactions

MS (left) and MS/MS (right) of L-TDP-*epi*-vancosamine **4**



MS (left) and MS/MS (right) of L-TDP-*epi*-vancoshydroxylamine intermediate **6**

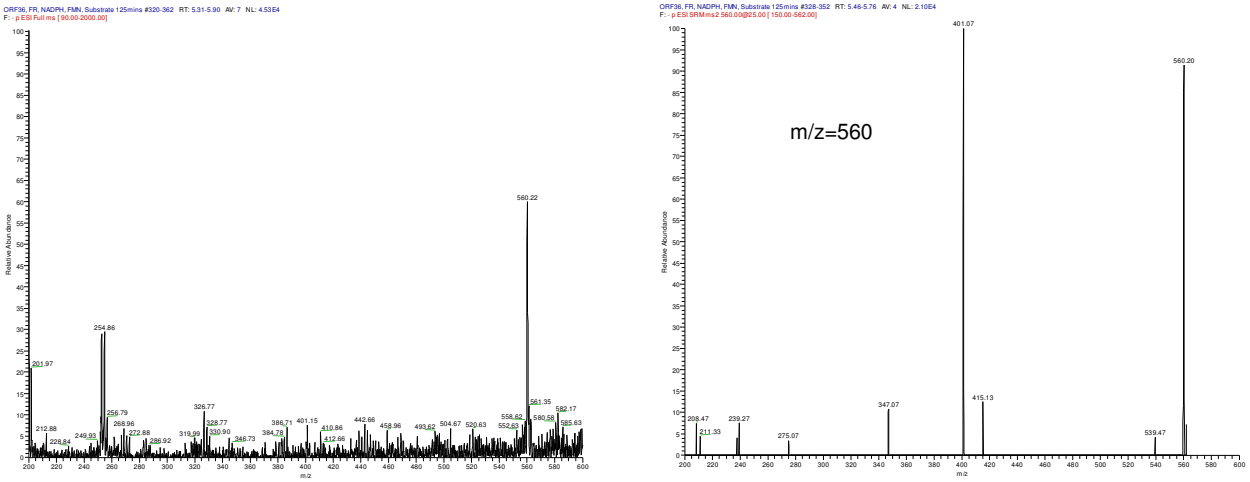
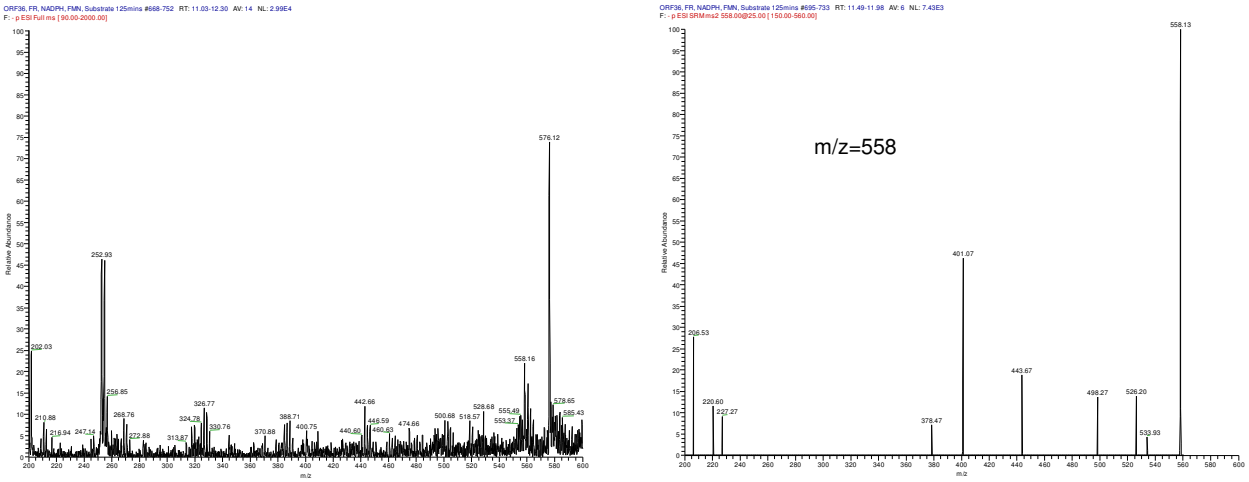
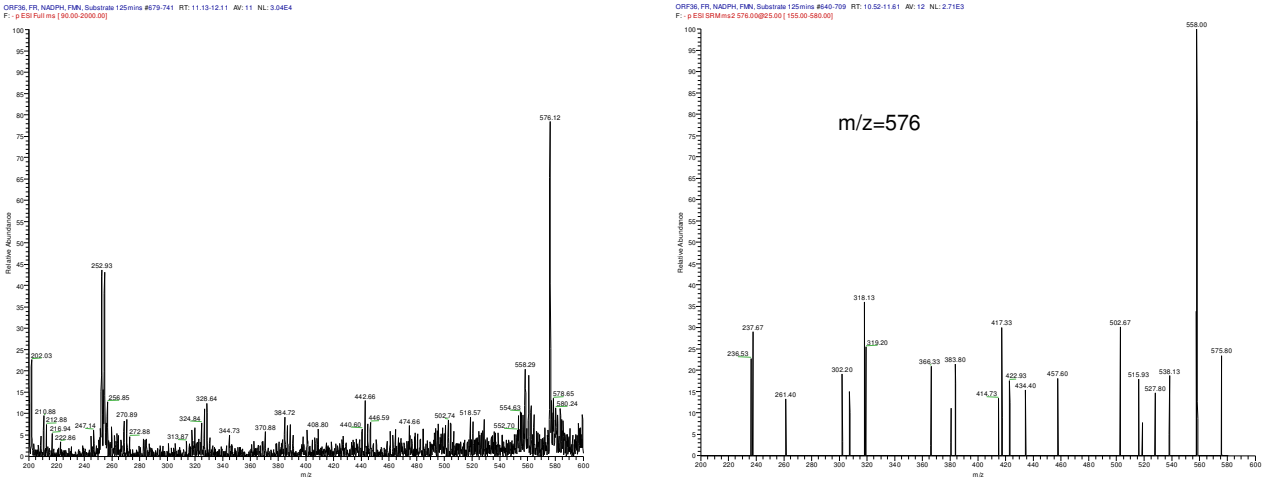


Figure S8 (con't). MS and Tandem MS of product ions for ORF36 catalyzed reactions

MS (left) and MS/MS (right) of L-TDP-*epi*-vanconitrose intermediate **7**



MS (left) and MS/MS (right) of L-TDP-epi-vanconitrose hydrate intermediate **8**



***Orf36* DNA sequence from *Micromonospora carbonacea* var *africana* (NRRL 15099) (Genbank locus accession AX574199):**

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CGTTCCCGCCGAGCTGGGCGGCCTGGGGTTGACCCGGCTGTACGACGTGGCGACCGC
GCTGATGCGGCTGGCCGAGGCCGACGCGTCCACCGCGCTGGCCTGGCACGTGCAGCT
CAGCCGGGGCCTCACCCTCACCTACGAGTGGCAGCACGGCACGCCGCCGGTGCGCGC
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ORF36 amino acid sequence (does not include His6 tag):

VAADLRAPLTPAGRTVVDLLAGVIPRISAEAADRDRTGTFPVEAFEQFAKLGLMGATVPAELGG
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QPYSYVDAVDYLSGQALGLDRDNDYMSVRALRSRTSA*

***rubN8* DNA sequence from *Streptomyces achromogenese var. rubradiris* (NRRL3061) (Genbank locus accession AJ871581):**

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CGCGCGCGACGGCGTGCTGGGCGCGACCGTGCCCGCCGAACTGGGAGGGATGGGCGTC
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CGCACTGGCCTTGCACGCCCAGTTCAGCCGGGGAATCACCCTGACATACGAGTGGCTGCA
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GAGGCGGTGATCGGCGGCGCGGTGAAGGACCACGGACGAGAGACCACCCGGCTGCGCC
CCGACGGCTCCGGTGGCTGGCTGCTGTCCGGACGCAAGACTCTGGTGACGATGGCGCCG
ATCGCGACGCACTTCGTCGTCTCCGCCCAGGCGCCGGCCGCCGGGGGAACAACGCTGCT
GTACGCCCCCATCGTGGCACGGGACACCCCGGGGCTCTCCATCGTCGACGGATGGACCG
GCCTGGGCATGCGGGCGTCCGGCACGCTCGACGTGGCGTTCGACGACTGCCCGGTGCC
CGCCGGAAACCTGCTGGCGCGTGGCAGCGTCGGCGCGCACAGCGACGCGGCCCTCGCC
GGGCAGGCGGTCAGCTCCGTCGCCATGCTCGGCATCTACGTCGGCGTCGCCCAGGCCGC
CCGCGACCTCGCGGTGGAGACCATGGCCCGGCGGTCGGCCACACCGCCCGCGGCCTCA
CGCACCCTGGTCGCCGAGACCGAGGCGCGGCTGTACGCGCTGCGGGCCACCGCATCGG
CTGCGCTCGTCAACGTCGACGAACTGTCGCCACGTCACGACATGGATCCCGATGAGCGCG
GCCGGCGGATGATGACCCCGTTCCAGTGCGCCAAAGTCATGGTCAACCAACTGGCGGCC
GCGGTGGTCGACGACTGCCTCACCGTGGTGGGCGGGGCCACCTACGCGGCGGAGCACC
CGCTGGCCCGGCTGTCCCGGGACGTGCGGGCCGGCCGGTTCATGCAGCCGTACACGTAC
GCCGACGGGGTCGACTACCTGAGCGCCCAGGCGCTCGGTTTGGAAAGAGACAACAACTA
CGTGAGCCTGCGGGCCACCCGGCCGGTGGACAGCCGGTGA

RubN8 amino acid sequence (Genbank accession: CAI94696) (does not include His6 tag):

METEQAPRPAEPPGDLTTAITAPGEQLLTLLDRHLPRIRAQAAPNDRDSTFPAATFHGFARDGV
LGATVPAELGGMGVSRLHDVAVALLRVAEADASTALALHAQFSRGITLTYEWLHGPPPTRKLA
ERLLRAMARGEAVIGGAVKDHGRETTRLRPDGSGGWLLSGRKTLVTMAPIATHFVVSAQAPAA
GGTTLLYAPIVARDTPGLSIVDGWTGLGMRASGTLDVAFDDCPVPAGNLLARGSVGAHSDAAL
AGQAVSSVAMLGIYVGVAQAARDLAVETMARRSATPPAASRTLVAETEARLYALRATASAALV
NVDELSPRHDMDPDERGRRMMTPFQCAKVMVNQLAAAVVDDCLTVVGGATYAAEHPLARLS
RDVRAGRFMQPYTYADGVDYLSAQALGLERDNNYVSLRATRPVDSR*