Enzyme-Catalyzed Synthesis of Furanosyl Nucleotides

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Assay Conditions

Enzymatic reactions containing 1.0 mM dTTP, 2.0 mM furanose-1-phosphate, 2.2 mM $MgCl_2$, and 0.5 EU inorganic pyrophosphatase were initiated by the addition of 10 EU Cps2L glucopyranosyl-1-phosphate thymidylyltransferase (*Streptococcus pneumoniae* R6) in Tris-HCl buffer (39 mM final buffer concentration, 50 μL reaction volume). The enzymatic reactions were conducted at 22 °C and reaction progress was monitored at 1, 6, 12, 18, and 24 h by HPLC. Enzymatic reaction aliquots (10 μL) were quenched with HPLC grade MeOH (10 μL) and centrifuged (5 min at 12,000 x *g*) to precipitate denatured enzymes prior to HPLC analysis.

Note: (i) that uncorrected percentage conversions refer to analysis of only NTP and sugar nucleotide product peak areas while corrected conversions take into account the degradation of sugar nucleotide products over the given time period (as determined by comparison with reactions containing no thymidylyltransferase under identical conditions). Assays were conducted at 22 °C instead of 37 °C to help minimize sugar nucleotide degradation.

(ii) that the HPLC method used to determine the conversions is documented in the Supporting Information of Timmons, S. C.; Mosher, R. H.; Knowles, S. A.; Jakeman, D. L. *Org. Lett.* **2007**, *9*, 857-860.

Kinetics Assay Conditions

(I don't understand the significance of this sentence. The enzymatic reaction conditions described below are actually different, so I think this statement is misleading.) 200 μL enzymatic reactions containing 1.0 mM dTTP, 2.2 mM $MgCl_2$, 2 EU inorganic pyrophosphatase, 100 μM thymidine and either 50 mM, 75 mM, 100 mM, 200 mM or 300 mM α-D-glucopyranose-1-phosphate or β-L-arabinofuranose-1-phosphate (accounting for the α/β mixture) were initiated by the addition of 0.102 μM Cps2L (0.001 EU) or 128 μM Cps2L (1.3 EU) for glucopyranose-1-phosphate and β-L-

arabinofuranose-1-phosphate, respectively. The enzymatic reactions were conducted at 37 °C and monitored at 1, 2, 4, 6, and 10 minutes (α-D-glucopyranose-1-phopshate) or 5, 10, 15, 30, and 45 minutes (β-L-arabinofuranose-1-phopshate). Enzymatic reaction aliquots (40 μL) were quenched with HPLC grade MeOH (40 μL) and centrifuged (5 min at 12,000 x *g*) to precipitate denatured enzymes prior to HPLC analysis. The HPLC method used to determine conversions is as described in the Supporting Information of Timmons, S. C.; Mosher, R. H.; Knowles, S. A.; Jakeman, D. L. *Org. Lett.* **2007,** *9*, 857-860 except using a linear gradient from 80:20 A/B to 75:35 A/B over 9.0 min to 40:60 A/B at 10.0 min followed by a plateau at 40:60 A/B from 10.0 to 11.0 min at 1.0 mL/min. Final concentrations of the sugar nucleotides were determined by comparing the product peak area to that of a thymidine internal standard (100 μM concentration in the reaction mixture). Initial rates were determined by plotting concentration of sugar nucleotides versus time. Michaelis-Menten and Lineweaver-Burk plots were fitted using GraFit 5.0 software.

Hydrophilic Interaction Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry (HILIC-ESI-MS/MS) Conditions

An Agilent 1100 LC system was coupled to the QTRAP 4000 mass spectrometer for the HILIC-ESI-MS/MS experiments. The samples were separated on a TSK-gel Amide 80 HILIC column (4.6 mm I.D. x 25 cm, Tosoh Bioscience) by gradient elution. The mobile phase comprised of an initial condition of MeCN: 6.5 mM ammonium acetate pH 5.5 80:20 (v/v) and was ramped linearly to 70:30 (v/v) in 10 min and then held at this composition for 5 min. A flow-rate of 1 mL/min was employed for the separations and reduced to 200 μL/min before introducing to the mass spectrometer. ESI-MS/MS analysis in the enhanced product ion (EPI) mode was performed on an Applied Biosystems-MDS SCIEX hybrid triple quadrupole linear ion trap (QTRAP 4000) mass spectrometer equipped with a Turbo V source for electrospray ionization. The mass spectrometer settings were: ionspray voltage

4.3 kV; mass range Q3 *m/z* 100–600; scan speed 1000 amu/sec; trap fill time was set to dynamic; Q1 and Q3 were set to unit resolution'. All acquisitions were made in the negative mode.

NMR Assay Conditions

Solutions containing 0.05 µmol dTTP, 0.1 µmol sugar-1-phosphate, and 0.11 µmol $MgCl_2$ were lyophilized. Enzymatic reactions were initiated by the addition of 20 EU Cps2L nucleotidylyltransferase in 20 µL Tris-d_{11}-DCl buffer (50 mM, pH 7.6, pH uncorrected for D_2O) for final reactant concentrations of 2.5 mM dTTP, 5.0 mM sugar-1-phosphate, 5.5 mM $MgCl_2$. The enzymatic reaction mixtures were incubated at 25 °C and analyzed using a Bruker Avance/DRX-500 high-resolution spectrometer with a MicroProbe located at the National Research Council Canada Institute for Marine Biosciences in Halifax.

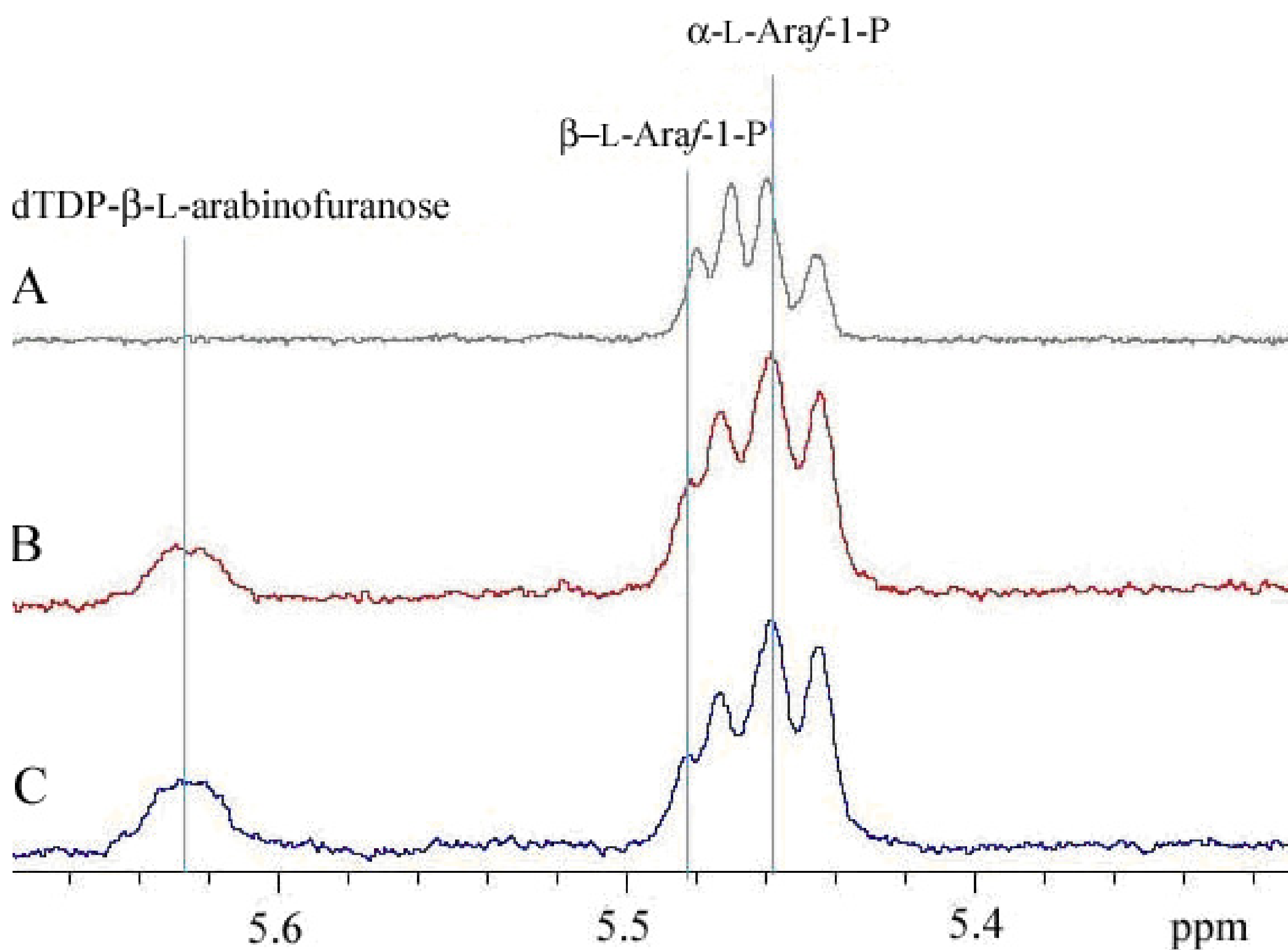


Figure S1. Nucleotidylyltransferase Cps2L is stereoselective for sugar-1-phosphate substrates

Spectrum A shows the reaction containing α/ß-L-arabinofuranosyl-1-phosphate prior to the addition of enzyme. Spectra B and C show the reaction mixture at 7 and 21 hours, respectively, illustrating the appearance of the anomeric signal for dTDP-α-L-arabinofuranose (5.62 ppm) in conjunction with the disappearance of the anomeric signal for β-L-Ara*f*-1-P (5.48 ppm) whilst the signal for α-L-Ara*f*-1-P (5.46 ppm) remains constant.

Table S1. Percentage conversions of dTTP to sugar nucleotide products using Cps2L

NTP	furanosyl-1-phosphate (α/β ratio)*	assay time (h)	% conversion‡ (uncorrected)	% conversion† (corrected)	dTDP-furanose retention time
dTTP	α/β-D-Gal*f*-1-P (1.2/1.0)	1	19	8	5.807
dTTP	α/β-D-Gal*f*-1-P (1.2/1.0)	6	36	22	5.821
dTTP	α/β-D-Gal*f*-1-P (1.2/1.0)	12	52	31	5.824
dTTP	α/β-D-Gal*f*-1-P (1.2/1.0)	18	63	33	5.821
dTTP	α/β-D-Gal*f*-1-P (1.2/1.0)	24	81	40	5.835
dTTP	α/β-L-Ara*f*-1-P (1.0/1.3)	1	47	39	5.913
dTTP	α/β-L-Ara*f*-1-P (1.0/1.3)	6	74	58 (66**)	5.911
dTTP	α/β-L-Ara*f*-1-P (1.0/1.3)	12	83	55	5.925
dTTP	α/β-L-Ara*f*-1-P (1.0/1.3)	18	89	49	5.937
dTTP	α/β-L-Ara*f*-1-P (1.0/1.3)	24	91	42	5.942
dTTP	α/β-D-Fuc*f*-1-P (1.5/1.0)	1	20	11	6.010
dTTP	α/β-D-Fuc*f*-1-P (1.5/1.0)	6	34	23	6.005
dTTP	α/β-D-Fuc*f*-1-P (1.5/1.0)	12	45	31	6.010
dTTP	α/β-D-Fuc*f*-1-P (1.5/1.0)	18	56	37	5.982
dTTP	α/β-D-Fuc*f*-1-P (1.5/1.0)	24	66	41	6.011
dTTP	6-Deoxy-6-Fluoro-α/β-D-Gal*f*-1-P (1.7/1.0)	1	16	8	6.012
dTTP	6-Deoxy-6-Fluoro-α/β-D-Gal*f*-1-P (1.7/1.0)	6	21	11	6.008
dTTP	6-Deoxy-6-Fluoro-α/β-D-Gal*f*-1-P (1.7/1.0)	12	29	14	6.012
dTTP	6-Deoxy-6-Fluoro-α/β-D-Gal*f*-1-P (1.7/1.0)	18	38	17	6.025
dTTP	6-Deoxy-6-Fluoro-a/b-D-Gal*f*-1-P (1.7/1.0)	24	45	19	6.015
dTTP	α/β-D-Glc*f*-1-P (1.9/1.0)	1	15	6	5.726
dTTP	α/β-D-Glc*f*-1-P (1.9/1.0)	6	19	6	5.725
dTTP	α/β-D-Glc*f*-1-P (1.9/1.0)	12	25	6	5.729
dTTP	α/β-D-Glc*f*-1-P (1.9/1.0)	18	34	7	5.747
dTTP	α/β-D-Glc*f*-1-P (1.9/1.0)	24	42	7	5.764

*Furanose-1-phosphates α/β-D-Gal*f*-1-P, α/β-D-Fuc*f*-1-P, and α/β-D-Glc*f*-1-P were synthesized according to the methods described in Euzen, R.; Ferrières, V.; Plusquellec, D. *J. Org. Chem.* **2005**, *70*, 847-855. The preparation of 6-Deoxy-6-Fluoro-α/β-D-Gal*f*-1-P and α/β-D-Fuc*f*-1-P were accomplished

in a similar manner and will be reported in due course elsewhere. **Accounting for α/β ratio. ‡Percentage conversion = ($A_P/(A_P+A_T)$) x 100, where A_P = dTDP-furanose product peak area and A_T = dTTP peak area. †Percentage conversion = ($A_P/(A_P+A_T+A_D)$) x 100, where A_P = dTDP-furanose product peak area, A_T = dTTP peak area, and A_D = degradation product peak area.

Please note that lines highlighted in yellow refer to the time points at which the highest corrected conversion to dTDP-furanose was observed. The HPLC traces for these time-points are presented below.

HPLC Traces for each Enzymatic Reaction Mixture

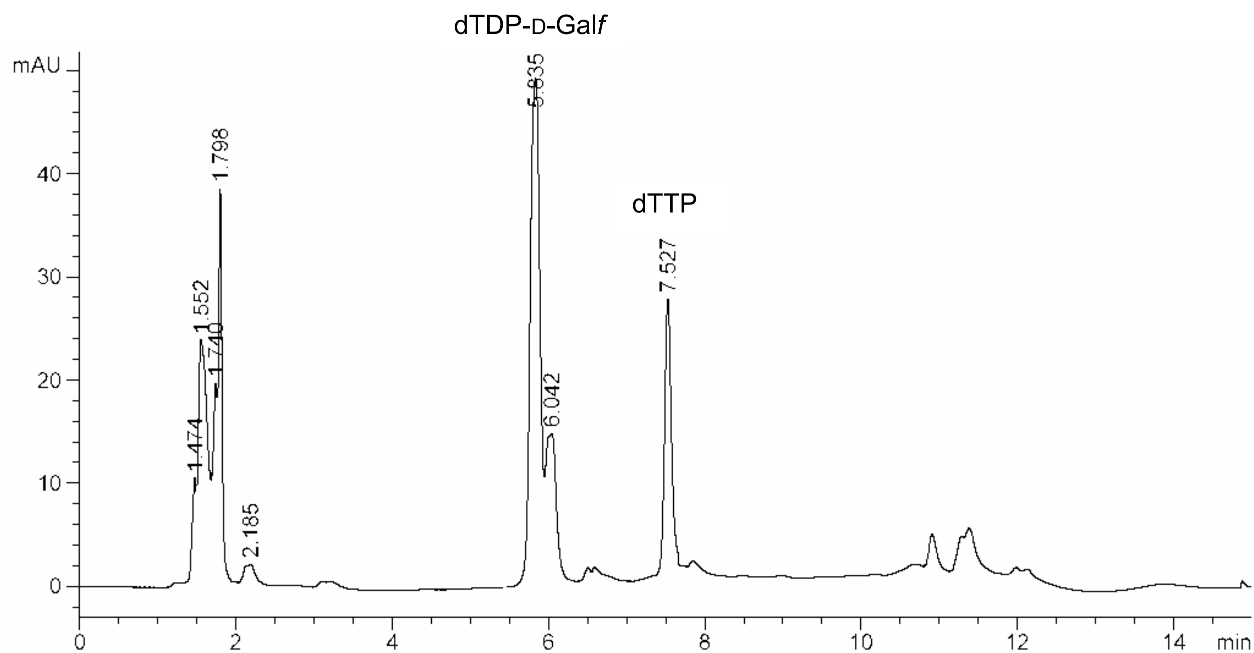


Figure S2. Cps2L + α/β-D-Gal*f*-1-P + dTTP after 24 h

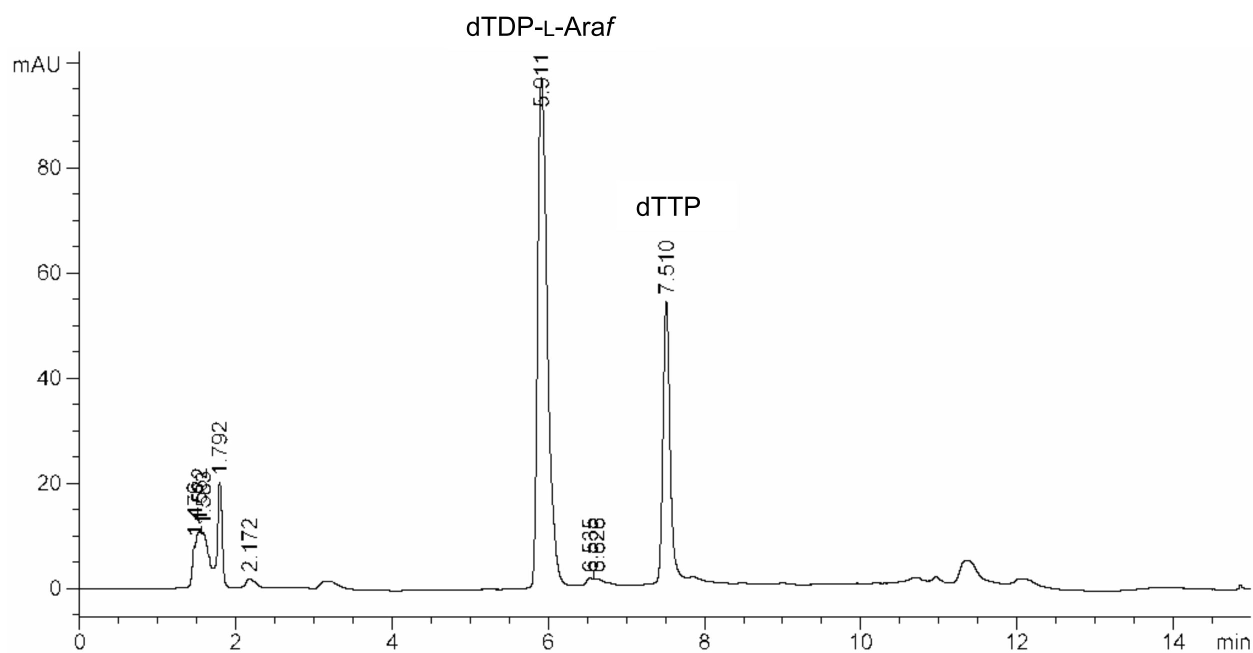


Figure S3. Cps2L + α/β-L-Ara*f*-1-P + dTTP after 6 h

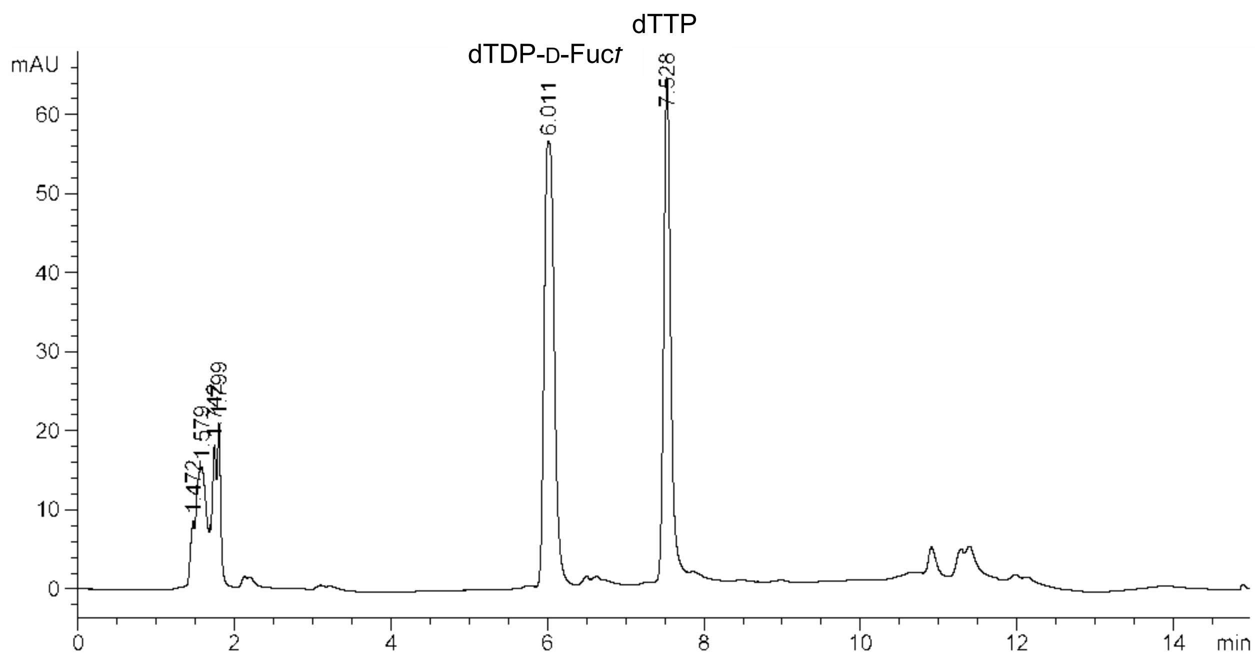


Figure S4. Cps2L + α/β-D-Fuc*f*-1-P + dTTP after 24 h

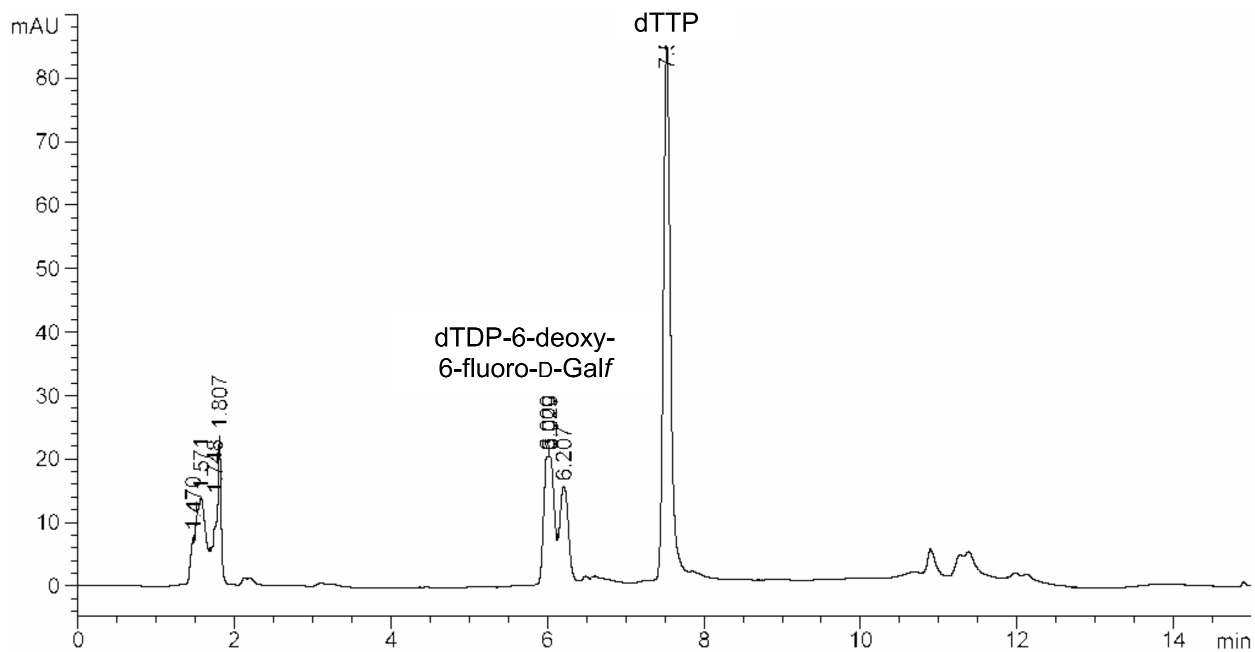


Figure S5. Cps2L + 6-Deoxy-6-Fluoro-α/β-D-Gal*f*-1-P + dTTP after 24 h

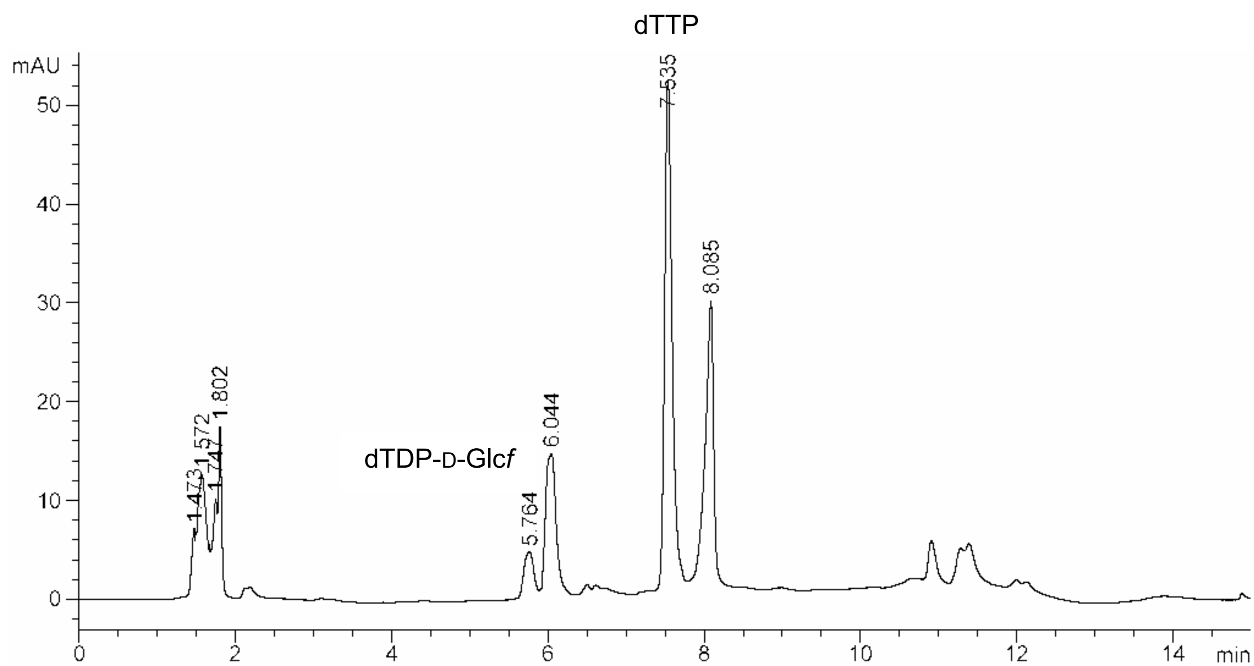
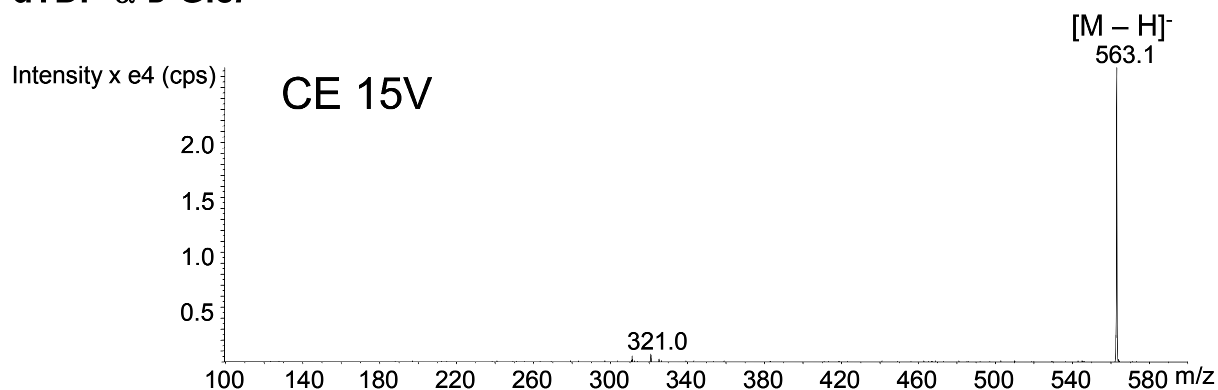
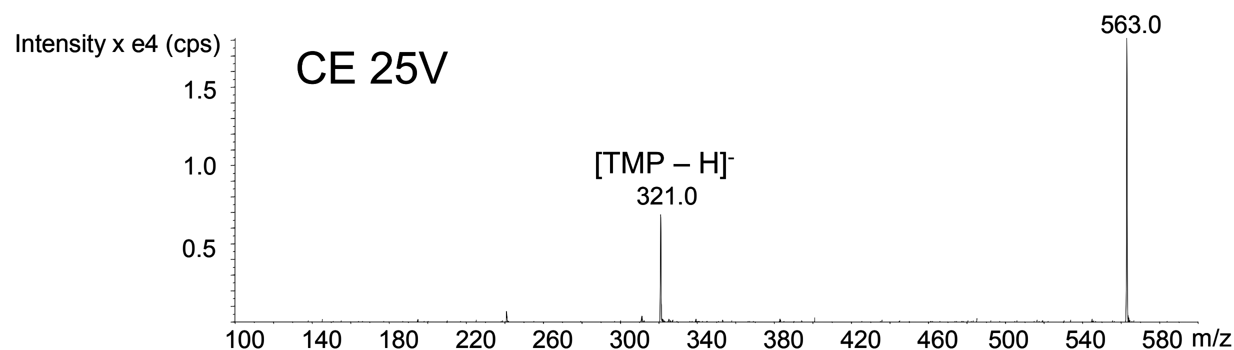


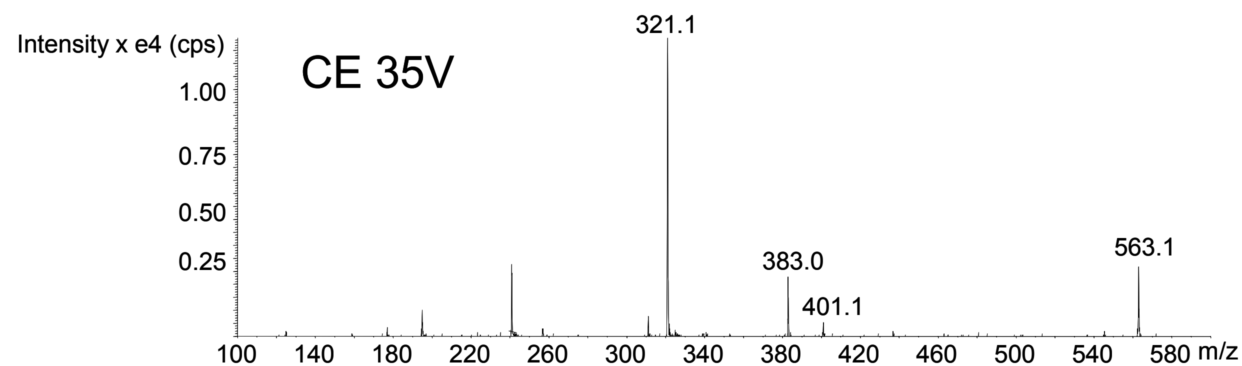
Figure S6. Cps2L + α/β-D-Glc*f*-1-P + dTTP after 24 h

HILIC-ESI-MS/MS EPI Scan Data of Enzyme-Catalyzed Preparation of Sugar Nucleotides

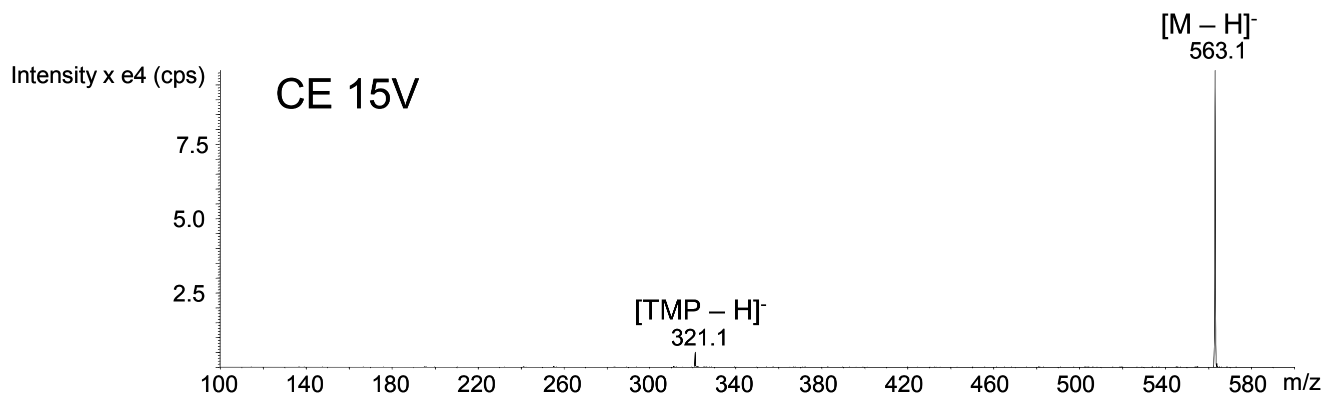
dTDP-α-D-Glc*f*

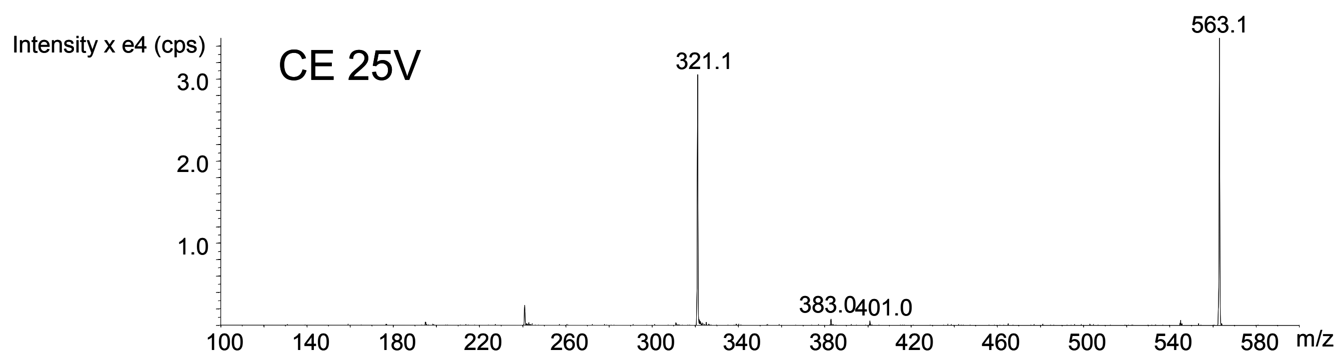


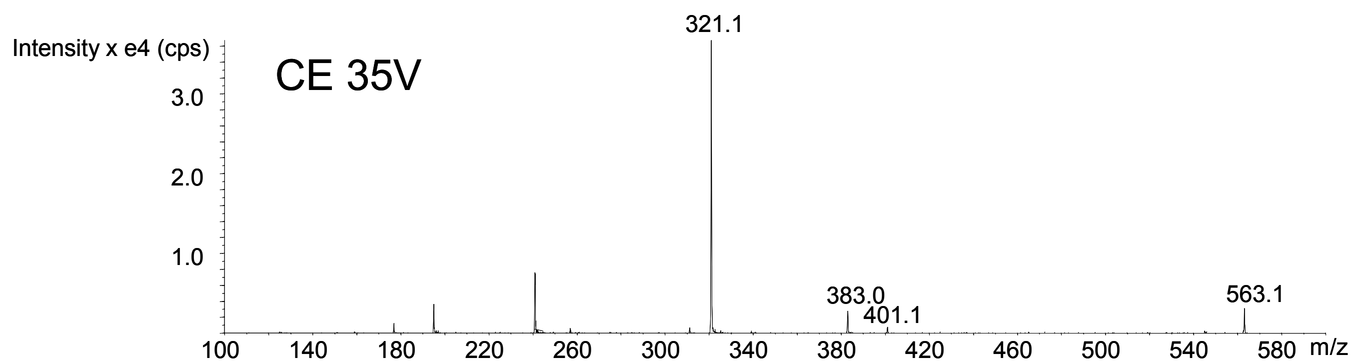




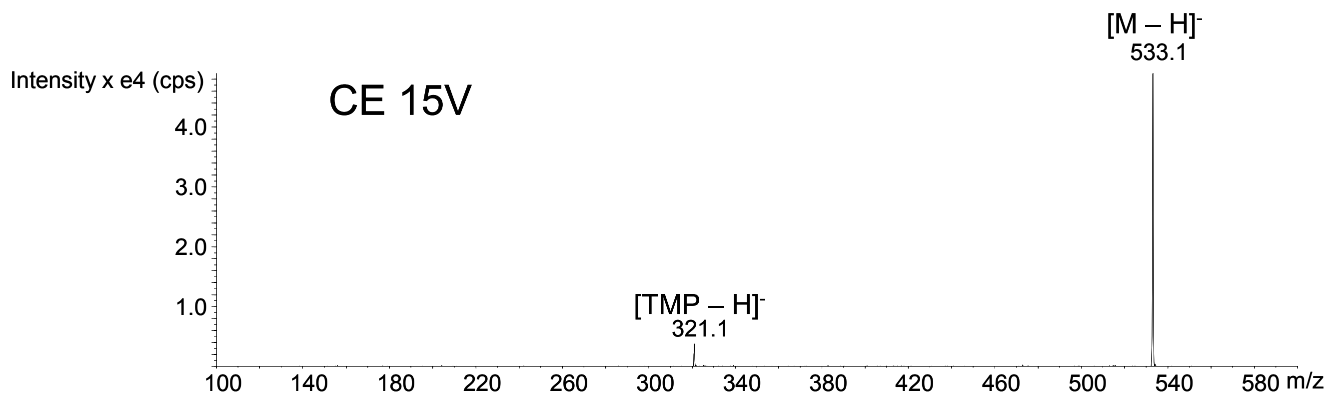
dTDP-α-D-Gal*f*

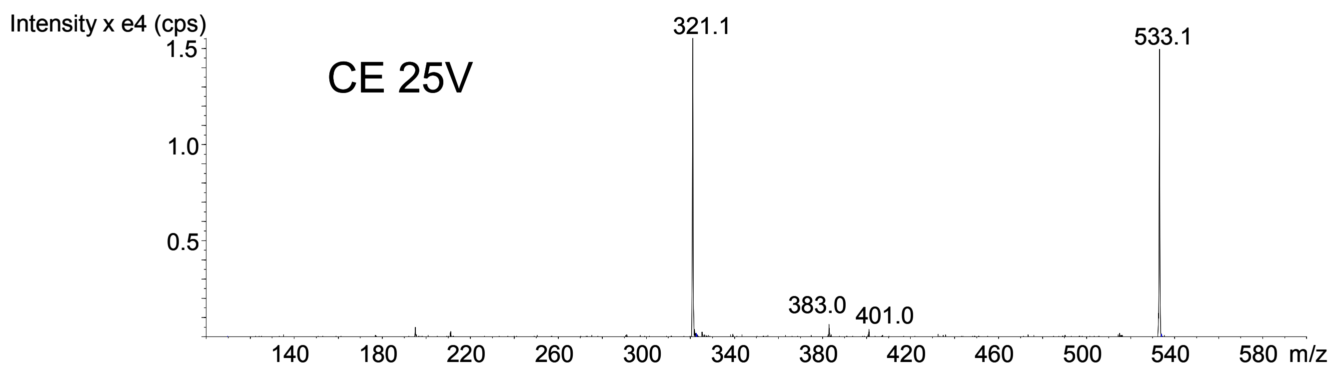


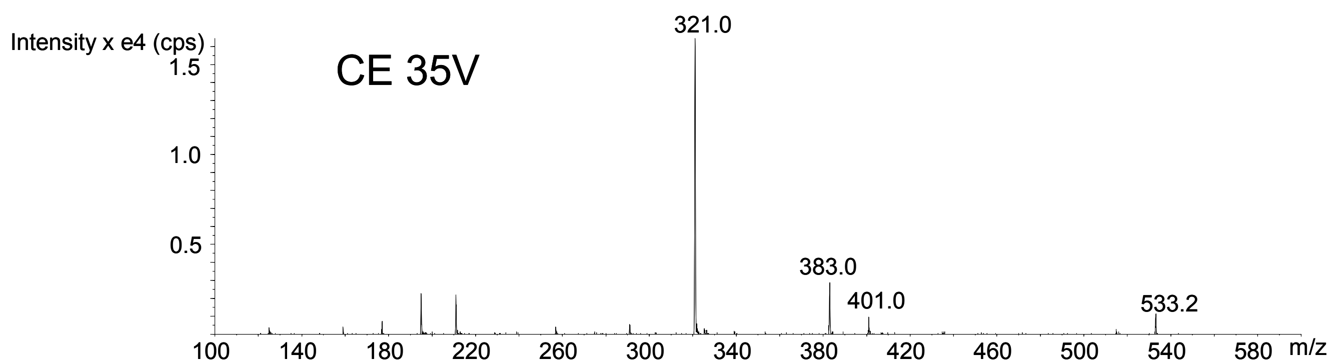




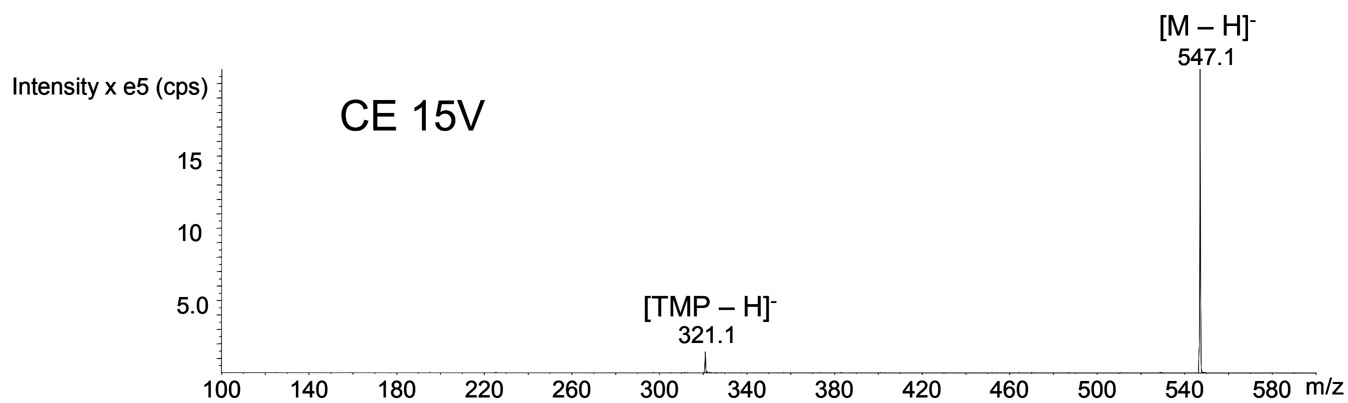
dTDP-β-L-Ara*f*

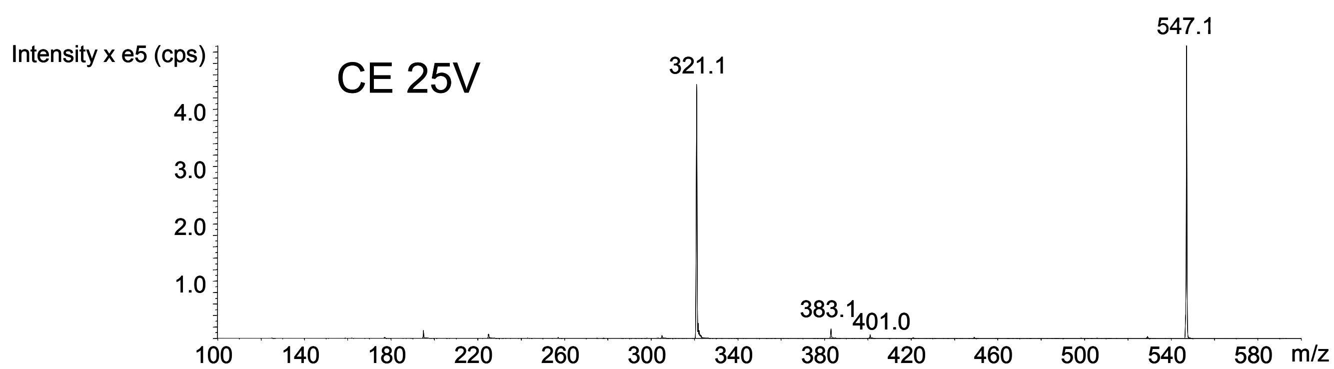


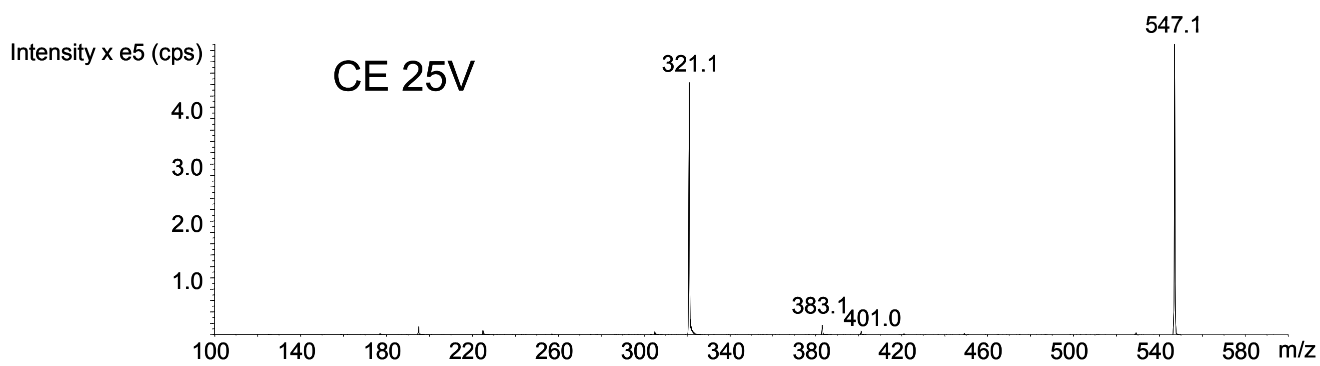




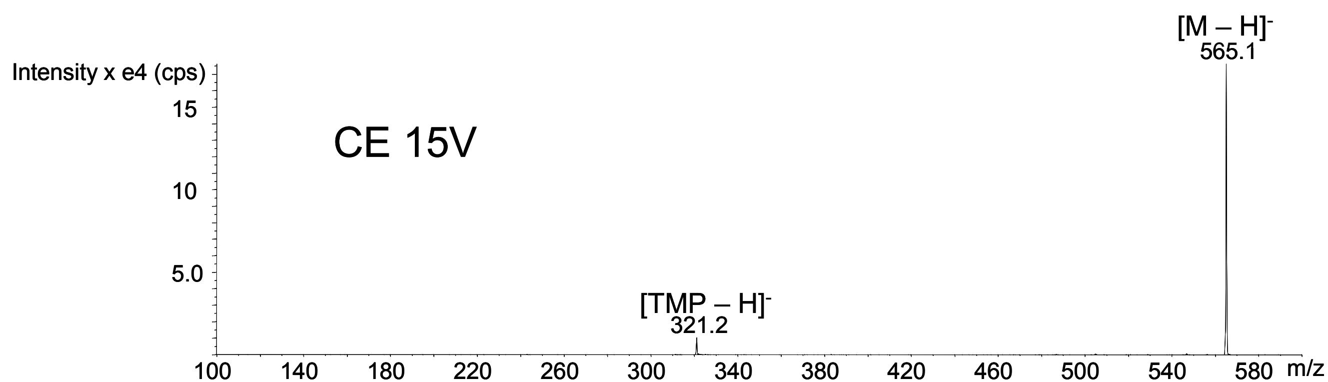
dTDP-α-D-Fuc*f*

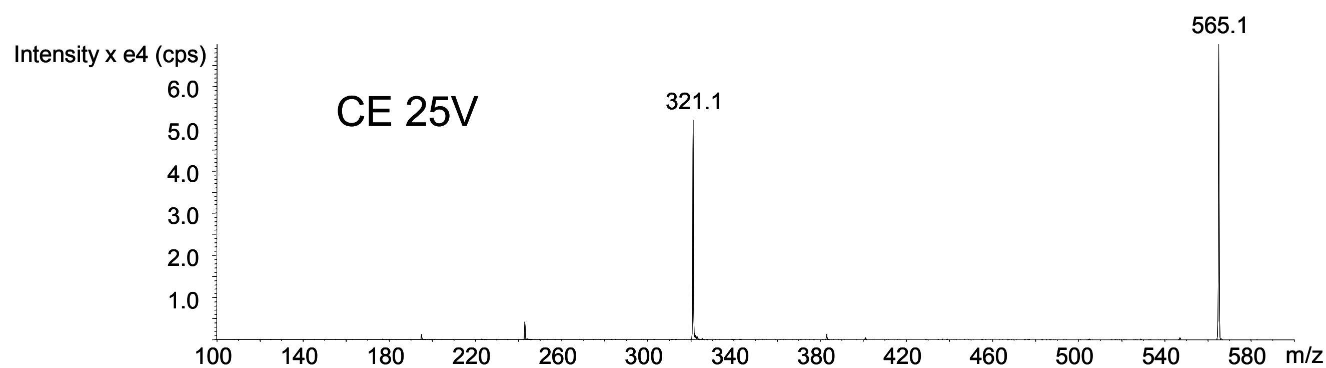


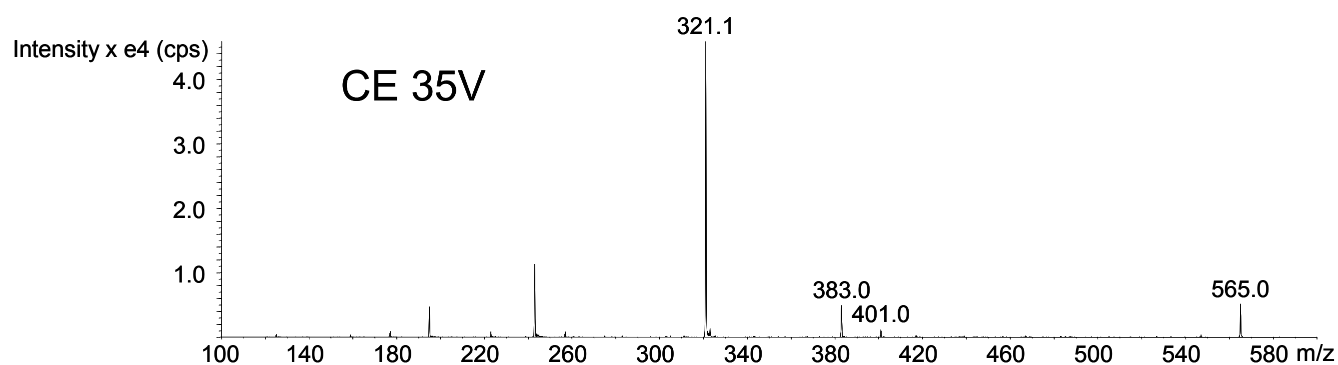




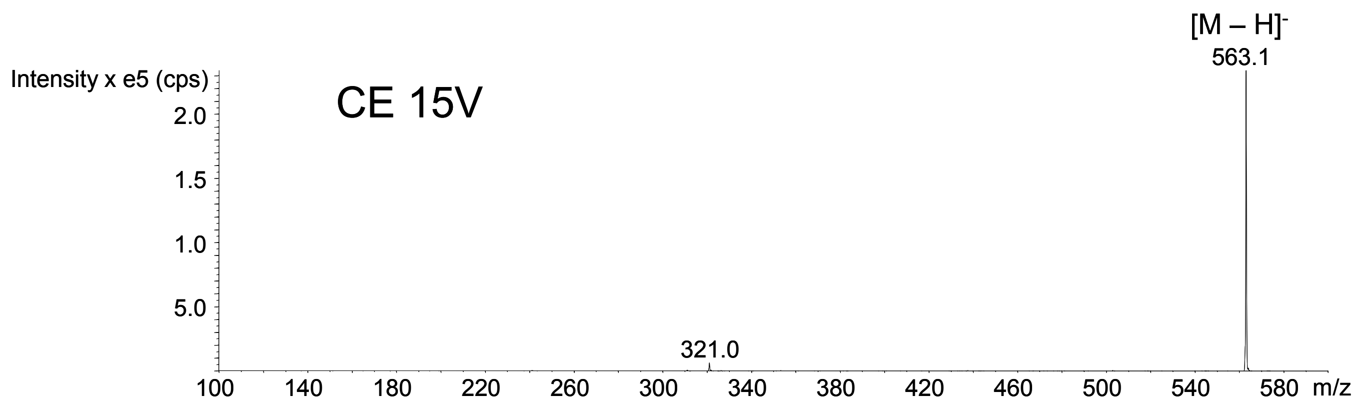
dTDP-α-D-6F-Fuc*f*

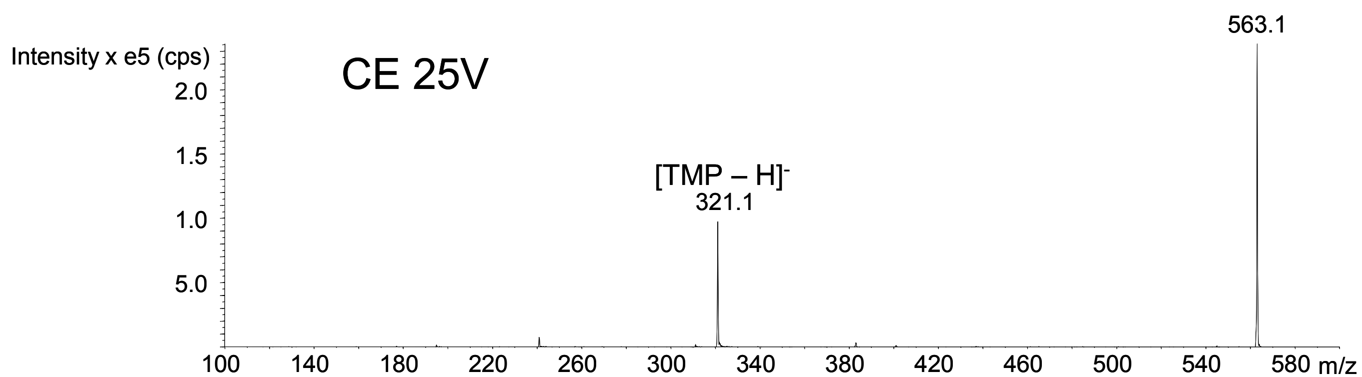


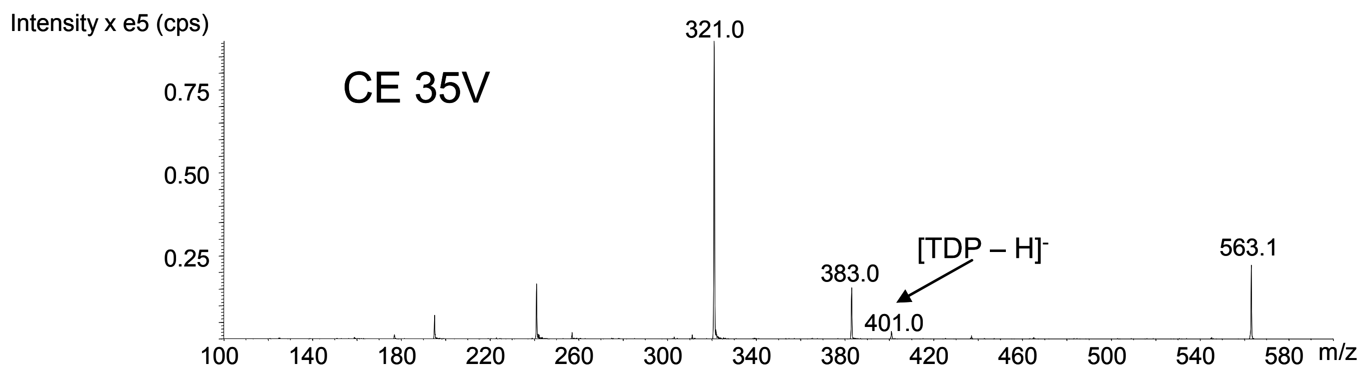




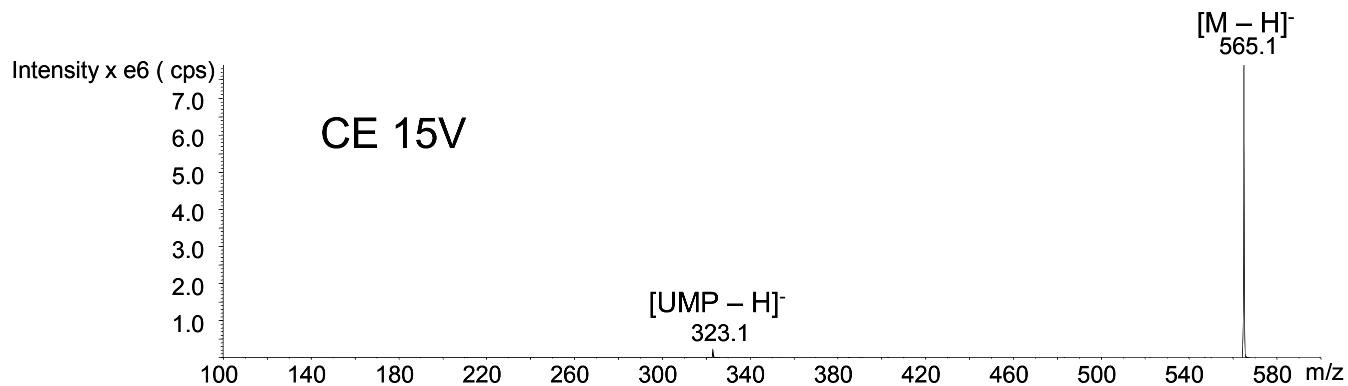
dTDP-α-D-Glc*p*

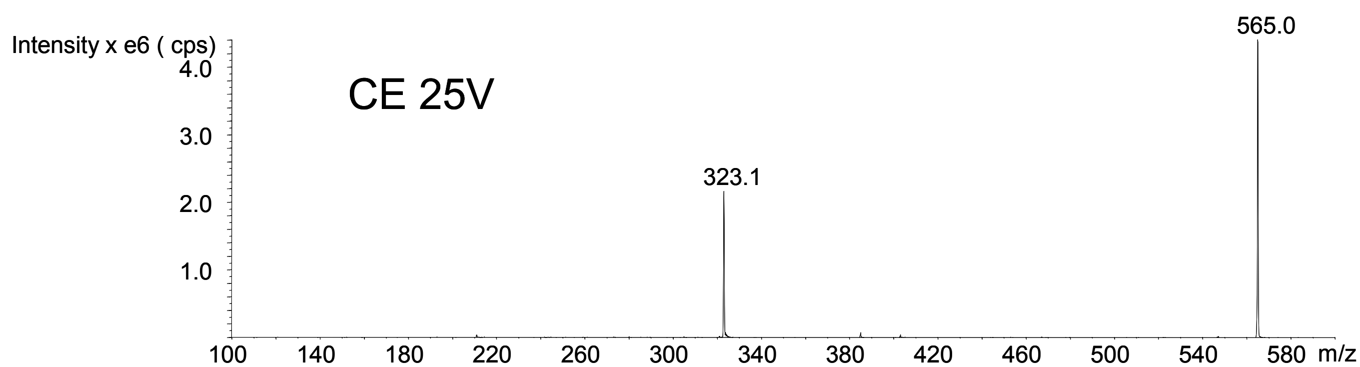


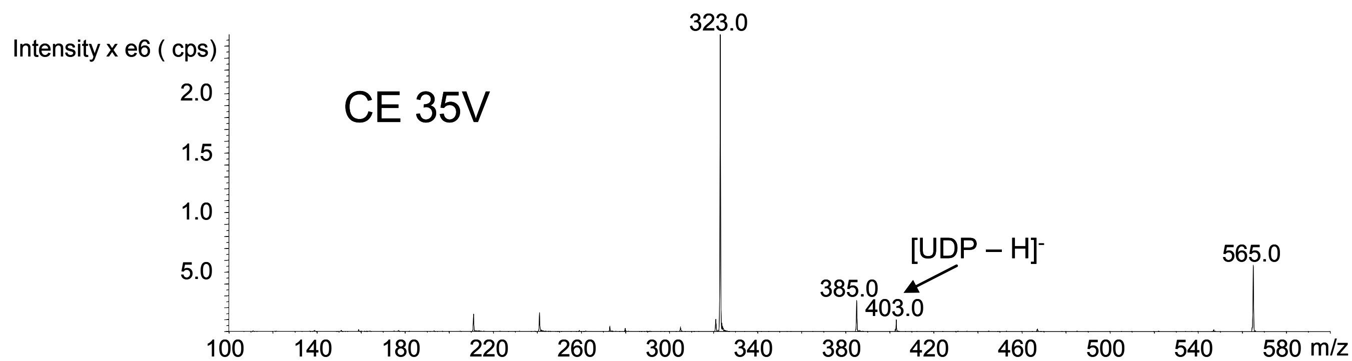




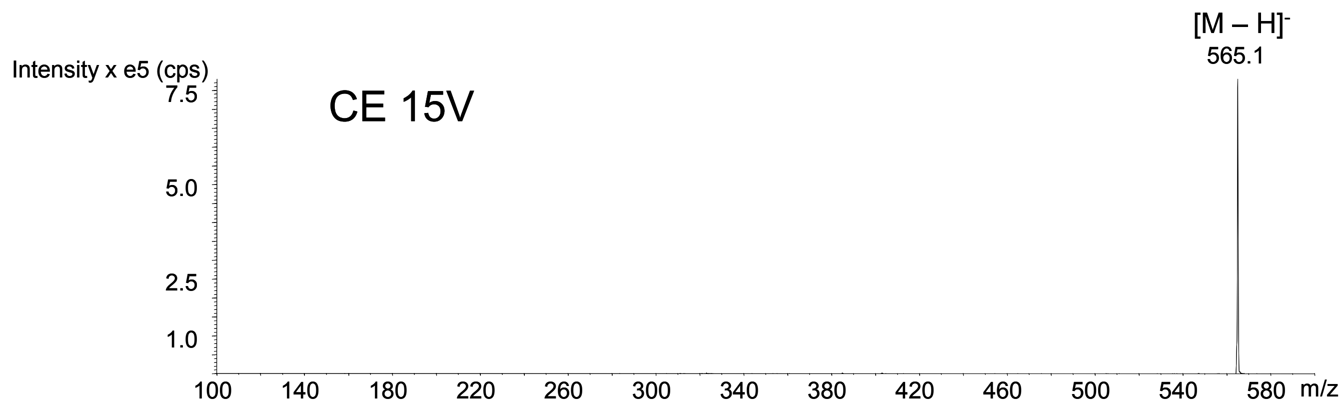
UDP-α-D-Glc*p*

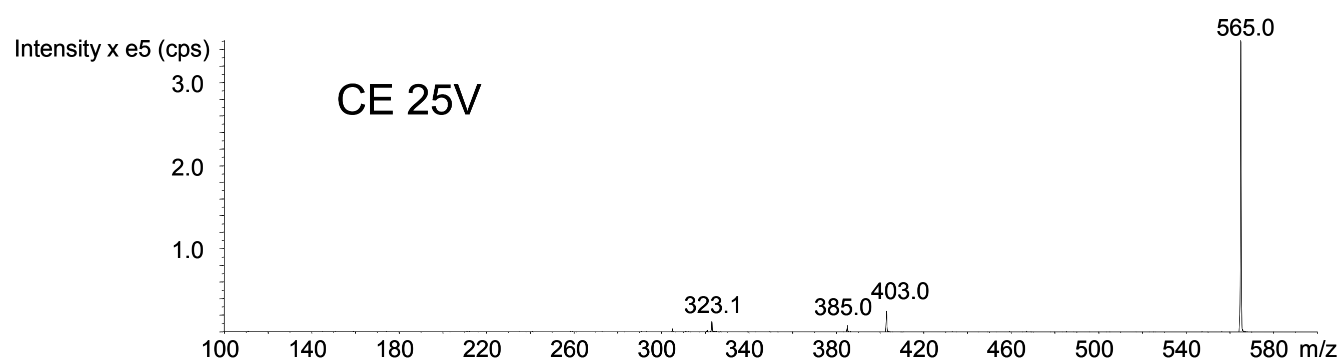


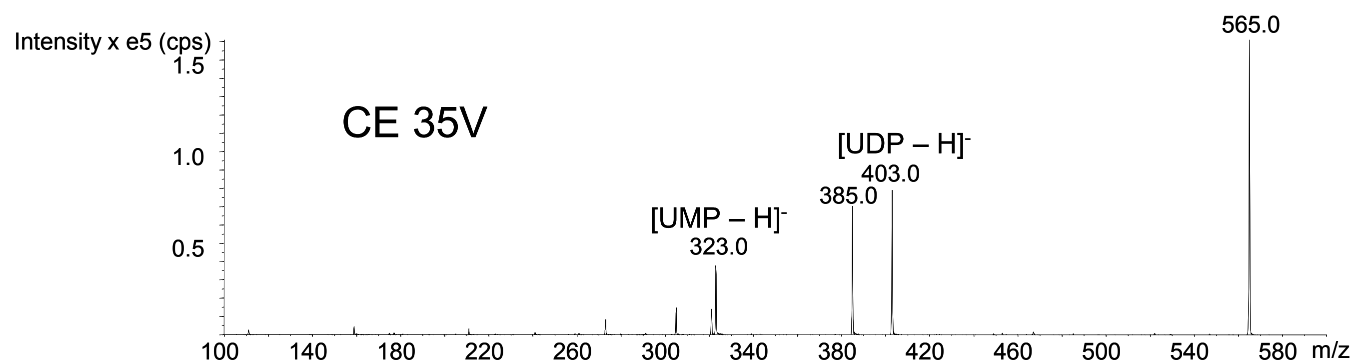




UDP-α-D-Man*p*







Kinetic parameters with respect to α-D-Glcp-1-P and β-L-Araf-1-P

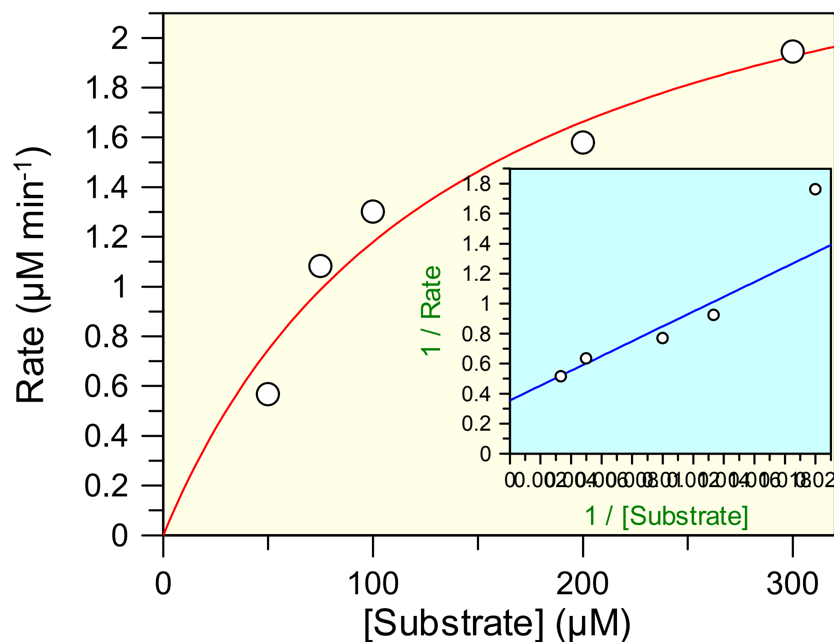


Figure S7. Michaelis-Menten and Lineweaver-Burk plots for Glc-1-P

Michaelis-Menton plot with inset Lineweaver-Burk plot demonstrating a V_{max} of 2.8 μM min^{-1} and a K_m of 139 μM with respect to α-D-Glc*p*-1-P.

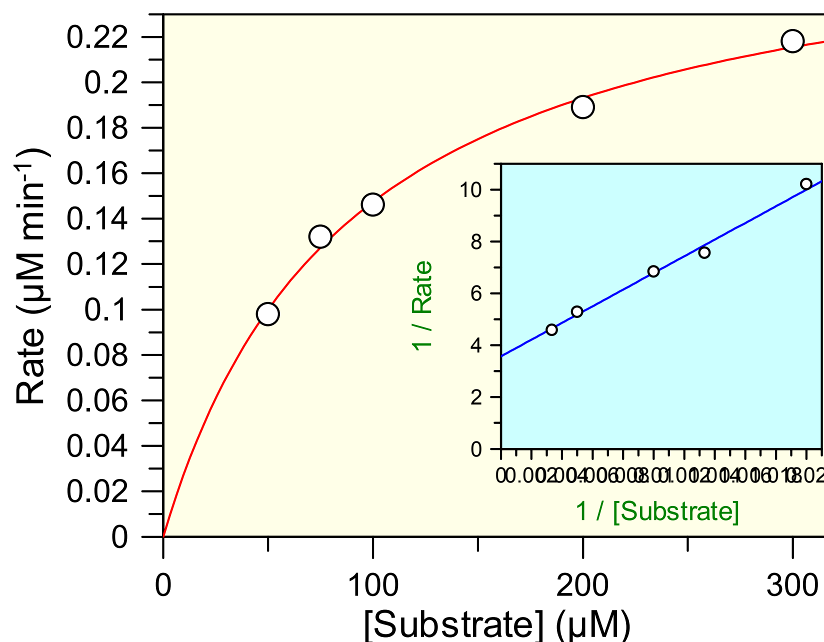


Figure S8. Michaelis-Menten and Lineweaver-Burk plots for Ara*f*-1-P

Michaelis-Menton plot with inset Lineweaver-Burk plot demonstrating a V_{max} of 0.28 µM min^{-1} and a K_m of 90.0 µM with respect to β-L-Ara*f*-1-P.

Table S2. Kinetic parameters for Cps2L with respect to α-D-Glcp-1-P and β-L-Araf-1-P

Parameter	α-D-Glc-1-P	β-L-Ara*f*-1-P
V_{max} (µM min^{-1})	2.8175	0.2801
K_m (µM)	138.9024	90.0493
k_{cat} (min^{-1})	27.6	0.00218
k_{cat}/K_m ($µM^{-1}$ min^{-1})	0.199	0.0000242