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Supporting Information

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Family 4 glycosidases carry out efficient hydrolysis of thioglycosides *via* an a,b-elimination mechanism

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Materials.

^{1}H NMR spectroscopy was performed on a Bruker Avance 400 spectrometer at 400 MHz. *p*-Nitrophenyl β-D-cellobioside was synthesized via the Koenigs-Knorr reaction.[1] Synthesis of *p*-nitrophenyl 4-deoxy-4-thio-6'-phospho-β-D-cellobioside (*S*-pNPC6′P) was achieved by a chemoenzymatic route involving the thioglycoligase methodology developed by Withers and co-workers as shown in Scheme 1, starting with *p*-nitrophenyl 4-deoxy-4-thio-D-glucoside.[2,3] The synthesis of *p*-nitrophenyl 4-deoxy-4-thio-D-glucoside will be published elsewhere. *p*-Nitrophenyl 4-deoxy-4-thio-6'-phospho-β-D-cellobioside was hydrolyzed using a cellulase from *Trichoderma viride* (obtained from Serva Electrophoresis) to yield 4-deoxy-4-thio-β-D-cellobiose, which was subsequently enzymatically phosphorylated to give the substrate 4-deoxy-4-thio-6'-phospho-β-D-cellobiose. The synthesis and characterization of 6'-phospho-β-D-cellobiose (*O*-C6′P) is as published in Yip et al.[4] Selective phosphorylation of the 6'-hydroxyl group on all compounds was accomplished enzymatically with BglK as described by Thompson[5] with minor modifications. All other chemicals were obtained from Sigma-Aldrich unless otherwise stated.

p-Nitrophenyl 6'-phospho-b-D-*cellobioside* (*O*-pNPC6'P)

103 mg, 80 %; **^{1}H NMR** (400 MHz, D_2O) δ 8.10 (2 H, d, $J_{Ar2,Ar3} = J_{Ar5,Ar6}$ 9.3 Hz, Ar3, Ar5), 7.08 (2 H, d, $J_{Ar2,Ar3} = J_{Ar5,Ar6}$ 9.3 Hz, Ar2, Ar6), 5.14 (1 H, d, $J_{1,2}$ 7.8 Hz, H1), 4.38 (1 H, d, $J_{1',2'}$ 7.9 Hz, H1′), 3.87-3.83 (3 H, m, $H6_a$, $H6_a'$, $H6_b'$), 3.71-3.34 (8 H, m, H2, H3, H4, H5, H3′, H4′, H5′), 3.22 (1 H, t, $J_{1',2'}$ 7.9 Hz, H2′); **^{13}C NMR** (100 MHz, D_2O) δ 161.54 (C), 142.48 (C), 125.98 (2 CH), 116.34 (2 CH), 102.79 (C1′), 99.04 (C1), 78.72, 75.35 (1 C, d, $J_{5',P}$ 7.3 Hz, C5′), 74.96, 74.87, 73.95, 73.25, 72.31, 68.84, 62.55

(1 C, d, $J_{6',P}$ 4.3 Hz, C6′), 59.75 (C6); **^{31}P NMR** (162 MHz, D_2O) δ 4.83 (1 P, t, $J_{H6a',H6b',P}$ 5.1 Hz); **ESI-MS** m/z: calc. for $[C_{18}H_{25}NO_{16}PNa_2]^+$ 588.0706; **Found**: 588.0707; **Anal. calc. For** $C_{18}H_{24}NO_{16}PNa_2{\cdot}4H_2O$: C, 32.79; H, 4.89; N, 2.12; Na, 6.97; O, 48.53; P, 4.70; **Found**: C, 32.54; H, 5.12; N, 2.17.

p-Nitrophenyl 4-deoxy-4-thio-6'-phospho-β-D-*cellobioside* (*S*-pNPC6'P)

61 mg, 76 %; **^{1}H NMR** (400 MHz, D_2O) δ 8.16 (2 H, d, $J_{Ar2,Ar3} = J_{Ar5,Ar6}$ 9.3 Hz, Ar3, Ar5), 7.14 (2 H, d, $J_{Ar2,Ar3} = J_{Ar5,Ar6}$ 9.3 Hz, Ar2, Ar6), 5.17 (1 H, d, $J_{1,2}$ 7.8 Hz, H1), 4.57 (1 H, d, $J_{1',2'}$ 9.9 Hz, H1′), 4.06-4.03 (1 H, m, $H6_a$), 3.94-3.79 (4 H, m, H5, $H6_b$, $H6_a'$, $H6_b'$), 3.65 (1 H, t, $J_{2,3} = J_{3,4}$ 9.3 Hz, H3), 3.58-3.52 (2 H, m, H2, H4′), 3.45-3.40 (2 H, m, H3′, H5′), 3.27 (1 H, t, $J_{1',2'} = J_{2',3'}$ 9.9 Hz, H2′), 2.88 (1 H, t, $J_{3,4} = J_{4,5}$ 10.5 Hz, H4); **^{13}C NMR** (100 MHz, D_2O) δ 161.57 (C), 142.44 (C), 126.01 (2 CH), 116.38 (2 CH), 99.01 (C1), 84.19 (C1′), 79.26 (1 C, d, $J_{5',P}$ 6.7 Hz, C5′), 76.51, 76.46, 73.84, 72.72, 72.55, 68.53, 62.58 (1 C, d, $J_{6',P}$ 4.4 Hz, C6′), 61.04 (C6), 47.18 (C4); **^{31}P NMR** (162 MHz, D_2O) δ 4.97 (1 P, t, $J_{H6a',H6b',P}$ 6.4 Hz); **ESI-MS** m/z: calc. for $[C_{18}H_{25}NO_{15}PNa_2S]^+$ 604.0478; **Found**: 604.0479; **Anal. calc. For** $C_{18}H_{24}NO_{15}PNa_2S{\cdot}3H_2O$: C, 32.88; H, 4.60; N, 2.13; Na, 6.99; O, 43.80; P, 4.71; S, 4.88; **Found**: C, 32.52; H, 4.84; N, 2.10.

4-Deoxy-4-thio-6'-phospho-β-D-*cellobiose* (*S*-C6'P)

9.3 mg, 40 %; **^{1}H NMR** (400 MHz, D_2O) δ 5.11 (1 H, d, $J_{\alpha1,\alpha2}$ 3.7 Hz, αH1), 4.51-4.47 (3 H, m, αH1′, βH1, βH1′), 3.96-3.93, 3.86-3.63, 3.58-3.52, 3.48-3.33, 3.22-3.16, 3.11 (1 H, t, J 8.5 Hz), 2.69 (1 H, t, $J_{3,4} = J_{4,5}$ 10.8 Hz, H4); **^{13}C NMR** (100 MHz, D_2O) δ 95.61 (βC1), 92.15 (αC1), 84.11 (α,βC1′), 79.49, 79.44, 79.38, 76.61 (2 CH), 76.25, 75.43, 72.96, 72.66 (2 CH), 71.48, 69.42, 68.76 (2 CH), 62.80 (α,βC6′), 61.47, 61.37, 47.70 (α,βC4); **^{31}P NMR** (162 MHz, D_2O) δ 5.28 (1 P, t, $J_{H6a',H6b',P}$ 7.1 Hz); **ESI-MS** m/z: calc. 483.0314, **Found**: 483.0315.

Enzyme kinetics.

All kinetic assays were conducted in 1 cm pathlength matched quartz cuvettes with a Cary 300 UV-vis spectrometer equipped with a circulating water bath, or a Cary 4000 UV-vis spectrometer with a

Cary Temperature Controller attached. Unless stated otherwise, BglT is preincubated in the assay buffer at 50 °C for 5 minutes prior to the addition of substrate to initiate the enzymatic reaction. All data fitting was performed with GraFit 4.0.

Glucose 6-phosphate dehydrogenase coupled assay.

Rates of hydrolysis of *O*-pNPC6′P, *S*-pNPC6′P, *O*-C6′P and *S*-C6′P were individually assayed using the glucose 6-phosphate dehydrogenase (G6PDH) coupled assay, as described in Yip et al.[4] 8-12 data points were collected for each substrate: *O*-pNPC6'P (final concentration range = 1-100 μM, final enzyme concentration = 45 μg/mL), *S*-pNPC6'P (final concentration range = 1-160 μM, final enzyme concentration = 22.5 mg/mL), *O*-C6′P (final concentration range = 10-1600 μM, final enzyme concentration = 5.63 μg/mL), and *S*-C6′P (final concentration range = 10-250 μM, final enzyme concentration = 5.6 mg/mL). A molar extinction coefficient of 6,220 $M^{-1}cm^{-1}$ for the oxidation of NADP to NADPH was used in calculations, and the catalytic parameters were determined based on a direct fit of the data to the Michaelis-Menten equation. Several controls were run to ensure that the coupled assay was meaningful. Firstly, the concentration of BglT was doubled for reaction at two different substrate concentrations and the observed rates were also doubled, ensuring that the BglT reaction was truly the rate-limiting process under study. Secondly, BglT was assayed with *p*-nitrophenyl 6-phospho-β-D-glucoside (*O*-pNPG6P) in the presence of 2 mM NADP, and the enzyme was also assayed with PNPG6P in the presence of 20 units of G6PDH. In each case the observed rate was the same as when BglT was assayed alone, indicating that the presence of NADP and G6PDH did not affect the activity of BglT.

Determination of K_i.

Kinetic studies were performed at 50 °C in 50 mM HEPES, 0.1 mM $MnCl_2$, 1 μM NAD^+, 10 mM 2-mercaptoethanol, and 0.1 % (w/v) BSA at pH 7.5, using 2.8 μg/mL BglT (final assay volume = 200 μL). Approximate K_i values were determined by measuring the reduction in BglT activity as measured by the hydrolysis of *O*-pNPG6P, in the presence of the disaccharide substrates. BglT was added to the assay mixtures containing a fixed concentration of *O*-pNPG6P and varying amounts of *O*-pNPC6′P, *S*-pNPC6′P, or *S*-C6′P. As the hydrolysis of the disaccharides does not release *p*-nitrophenolate anion, the

disaccharides can be considered as competitive inhibitors against the *O*-pNPG6P substrate, and the rate of hydrolysis of *O*-pNPG6P can be monitored spectrophotometrically at 400 nm. The experiments were repeated at different concentrations of *O*-pNPG6P. The data was graphed on a Dixon plot ($1/v$ vs [competitive inhibitor]). A horizontal line drawn through $1/V_{max}$ in the Dixon plot intersects the experimental lines at an inhibitor concentration equal to $-K_i$.

Abg coupled assay.

Rates of hydrolysis of *O*-pNPC6'P by BglT were also determined by use of the GH1 β-glucosidase, Abg as a coupling enzyme. All experiments were carried out at 50 °C in 50 mM HEPES buffer, pH 7.5, 0.1 mM $MnCl_2$, 1 μM NAD, 10 mM 2-mercaptoethanol, 8.5 μg/mL Abg and 0.1 % (w/v) BSA. BglT activity was measured spectrophotometrically by monitoring the release of *p*-nitrophenolate anion at 400 nm. BglT (final concentration of 90 μg/mL) was preincubated with the above solution at 50 °C, and the initial linear rate of increase in absorbance at 400 nm is measured upon addition of *O*-pNPC6'P (concentration ranging from 1-350 μM) to a final assay volume of 200 μL. The difference in extinction coefficients, Δε, between *p*-nitrophenyl β-D-glucoside and the *p*-nitrophenolate anion at pH 7.5, 50 °C was determined to be 13,791 $M^{-1}cm^{-1}$, and the catalytic parameters were determined based on a direct fit of the data to the Michaelis-Menten equation. The concentration of BglT was doubled for two data points and the observed rate was also doubled, ensuring that the concentration of Abg used was not the rate-limiting factor. Furthermore, BglT was assayed with *O*-pNPG6P in the presence of Abg and the observed rate was the same as when BglT was assayed alone, indicating that Abg did not hydrolyze 6-phospho-β-D-glucosides and that the presence of Abg did not affect the activity of BglT.

Solvent Deuterium Isotope Exchange. All buffers and chemicals were lyophilized twice from 99.9 % D_2O. BglT was exchanged into deuterated buffer solutions via repeated (three times) dilution and concentration using a centrifugal filter unit (Millipore) with a nominal molecular weight limit (NMWL) of 30 kDa. The enzymatic reactions were performed under the following conditions: 5 mM HEPES pH 7.5, 0.1 mM $MnCl_2$, 1 μM NAD^+, 0.4 mg/mL BglT. *S*-C6′P (1 mg) was incubated at 50 ºC in 5 mL of the above solution and the reaction was monitored by TLC. Upon completion, the enzyme was removed

using a 30 kDa NMWL centrifugal filter unit. 10 mg of Chelex 100 resin was added to the filtrate and stirred at room temperature for 30 minutes. The Chelex resin was removed via filtration, and the solution was lyophilized then redissolved in D_2O for ^1H NMR and MS analyses.

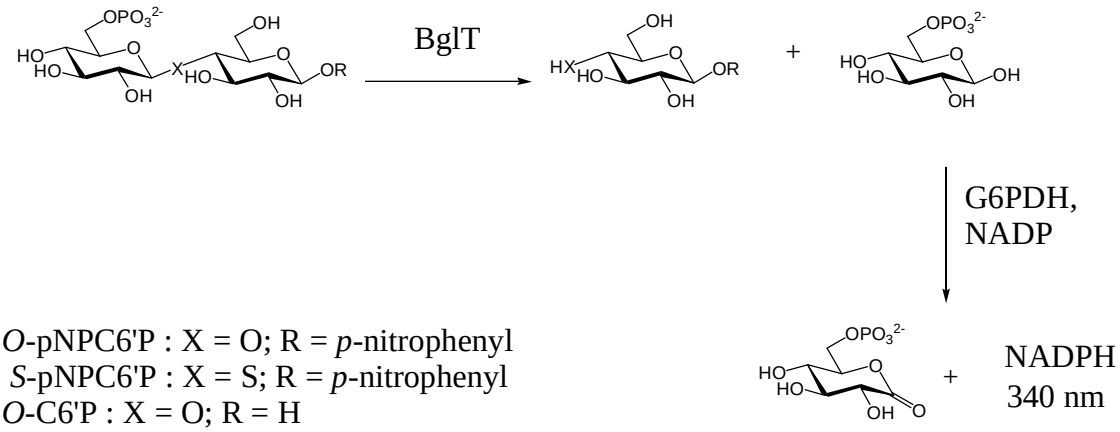
(1) Conchie, J., Levvy, G. A. *Methods in Carbohydrate Chemistry II* **1964**, *335*.

(2) Jahn, M., Marles, J., Warren, R. A. J., Withers, S. G. *Angew. Chem. Int. Ed.* **2003**, *42*, 352-354.

(3) Mullegger, J., Jahn, M., Chen, H., Warren, R. A. J., Withers, S. G. *Protein Eng. Des. Sel.* **2005**, *18*, 33-40.

(4) Yip, V. L. Y., Withers, S. G. *Biochemistry* **2006**, *45*, 571-580.

(5) Thompson, J., Lichtenthaler, F. W., Peters, S., Pikis, A. *J. Biol. Chem.* **2002**, *277*, 34310-34321.



O-pNPC6'P : X = O; R = *p*-nitrophenyl
S-pNPC6'P : X = S; R = *p*-nitrophenyl
O-C6'P : X = O; R = H
S-C6'P : X = S; R = H

Figure S1: G6PDH coupled assay.

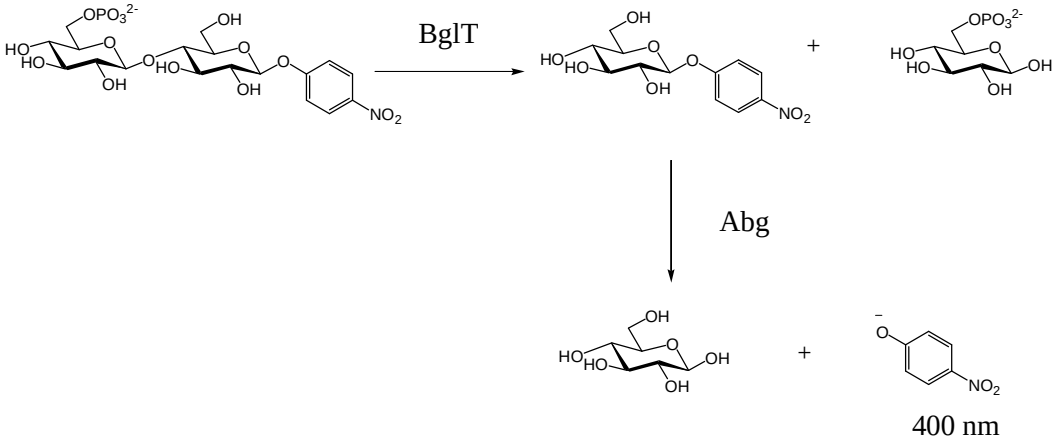
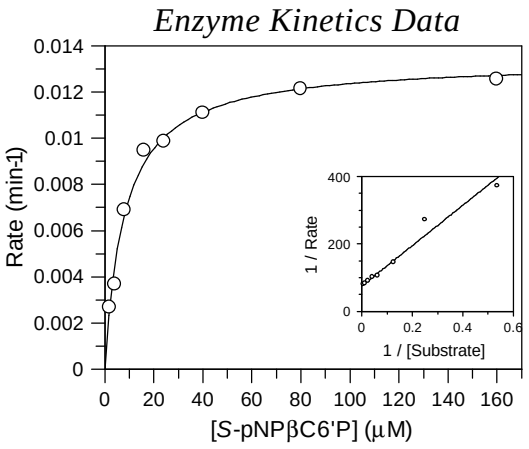


Figure S2: Abg coupled assay.



Parameter	Value	Std. Error
Vmax	0.0134	0.0003
Km	7.9994	0.7802

Figure S3: Plot of reaction rate *vs* substrate concentration for the reaction of BglT with *S*-pNPC6′P.

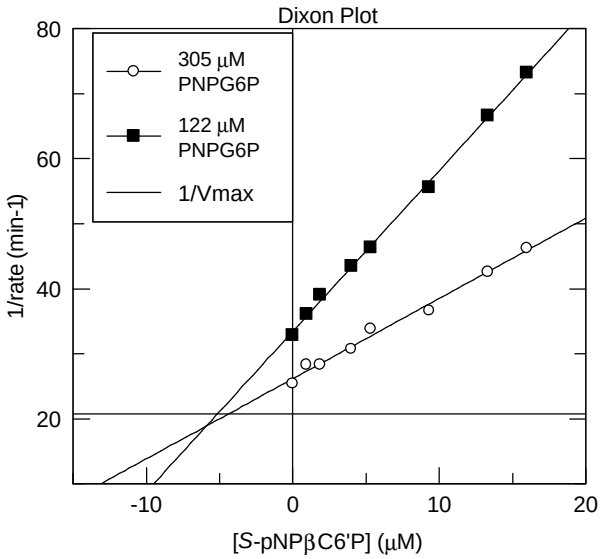
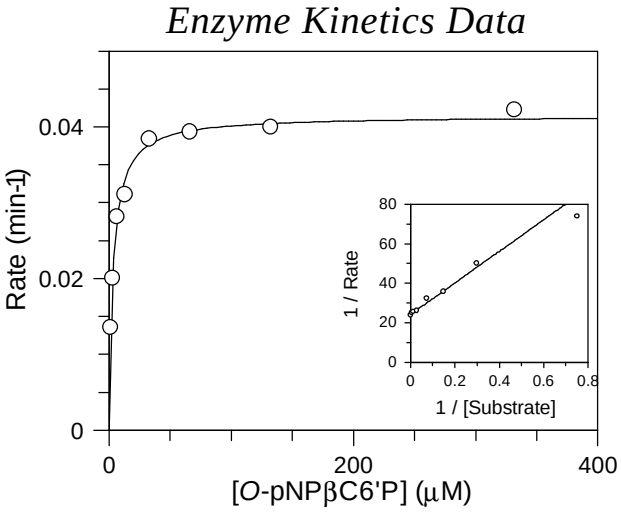
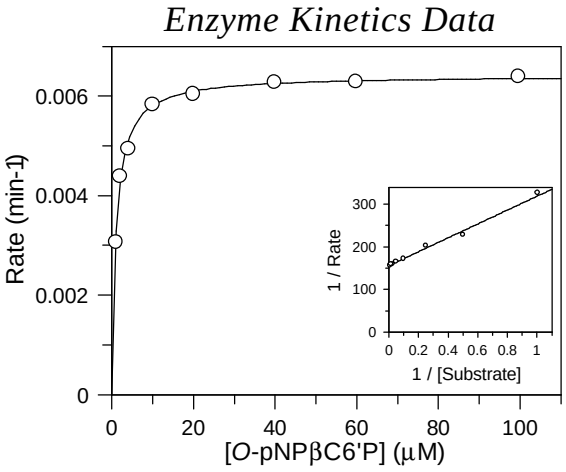


Figure S4: Dixon plot of *S*-pNPC6′P.



Parameter	Value	Std. Error
Vmax	0.0414	0.0007
Km	3.3139	0.3042

Figure S5: Plot of reaction rate *vs* substrate concentration for the reaction of BglT with *O*-pNPC6′P (Abg coupled assay).



Parameter	Value	Std. Error
Vmax	0.0064	5.30373e-005
Km	1.0440	0.0540

Figure S6: Plot of reaction rate *vs* substrate concentration for the reaction of BglT with *O*-pNPC6′P (G6PDH coupled assay).

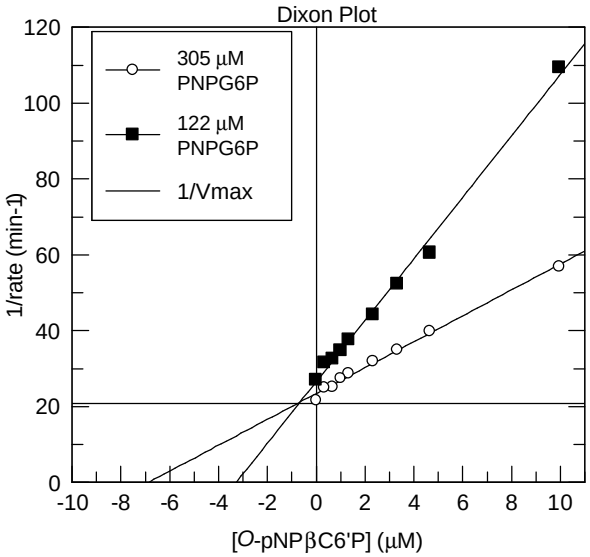
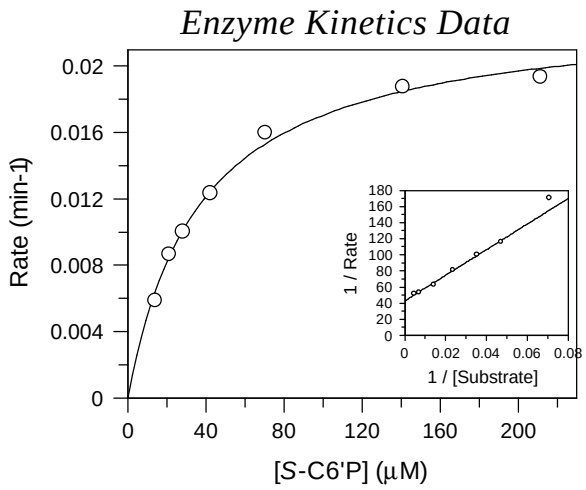


Figure S7: Dixon plot of *O*-pNPC6′P.



Parameter	Value	Std. Error
Vmax	0.0233	0.0006
Km	36.9822	2.8213

Figure S8: Plot of reaction rate *vs* substrate concentration for the reaction of BglT with *S*-C6′P.

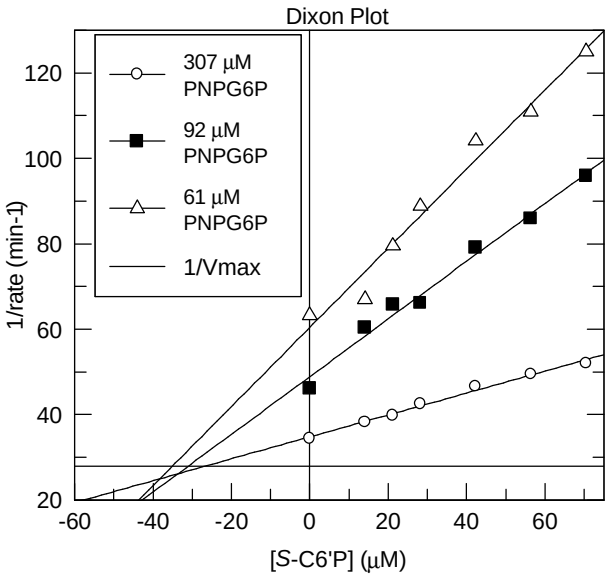


Figure S9: Dixon plot of *S*-C6′P.