Facile Formation of β-thioGlcNAc Linkages to Thiol-Containing Sugars, Peptides, and Proteins using a Mutant GH20 Hexosaminidase

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1) Experimental Procedures

Materials and reagents
All reagents and solvents were obtained from commercial suppliers and used without further purification. Analytical thin layer chromatography (TLC) was performed using Merck TLC silica gel 60-F254 plates and visualized by UV and a p-anisaldehyde stain.

Protein expression and purification
The plasmid construction as well as expression and purification of SpHex WT, SpHex D313A and SpHex E314A was performed, as previously reported.[1] E. coli BL21 (DE3) was chosen as expression host. 500 mL of 2xYT medium containing 100 µg/mL ampicillin was inoculated with resuspended cells from a 5 mL overnight culture. Expression cultures were grown at 25°C until A600 = 0.5-0.7 at which point protein production was induced by addition of IPTG to a final concentration of 0.4 mM, and incubation was continued for 16h. Cells were harvested by centrifugation at 8000 rpm for 30 min at 4 °C in a Beckman Coulter Avanti® J-E floor centrifuge (JA-10 rotor) and the pellet was stored at -70 °C until needed. The cell pellets were thawed in an ice bath and resuspended in 20 mL of lysis buffer (20 mM Tris, pH 8, 300 mM NaCl, 1 mM 2-mercaptoethanol, 0.3 mg/mL lysozyme, 0.625 U/mL benzonase, 10 mg/mL MgCl2, and 1 Pierce Protease Inhibitor mini tablet/40 mL). Afterwards the cells were lysed by sonication and the cell debris was removed by centrifugation (15000 rpm, 30 min, 4 °C, JA-20 rotor). The soluble fraction was purified by immobilized nickel affinity chromatography (1 mL HisTrap FF column (GE Healthcare)) on a GE Healthcare ÄKTA FPLC equipped with a UV detector (280 nm) and an automatic fraction collector. The samples were loaded at 1 mL/min on the HisTrap FF column and washed with loading buffer (20 mM Tris, pH 8, 1 mM 2-mercaptoethanol, 500 mM NaCl, 20 mM imidazole). The column was eluted with 10 mL of 95% Buffer A (20 mM Tris, pH 8, 300 mM NaCl, 1 mM 2-mercaptoethanol): 5% Buffer B (Same as A but also contains 500 mM imidazole), followed by a linear gradient over 25 mL to 50% A:50% B, and finally a linear gradient over 5 mL to 100% B. Fractions were analyzed by SDS-PAGE; protein containing fractions were combined, concentrated and exchanged to buffer A using an Amicon® Ultra-4 MWCO10 kDa centrifugal filter (Merck).

The C-terminal half of tau (244-441) was designed as a triple mutant replacing the two native cysteines by serine (C301S and C342S) while changing the GlcNAcylation site to a cysteine (S400C). The gene was cloned in a pET-28 vector and afterwards transformed and expressed in E. coli BL21 Rosetta 2 (DE3), inducing expression with 1 mM IPTG. Cells were harvested by centrifugation and resuspended in lysis buffer (300 mM NaCl, 0.25 mM 1,4-dithiothreitol (DTT), 1 % Triton X100, 5 mM imidazole, 50 mM sodium phosphate, pH 8) containing a Pierce protease inhibitor tablet and lysozyme (6 mg/mL). The suspension was heated to 75°C for 30 min prior to sonication. The cell debris was removed by centrifugation and tau isolated by nickel affinity chromatography as described above.

Kinetic analysis
Kinetic studies were performed at 37°C in citrate/phosphate buffer (25/25 mM, 100 mM NaCl, 2 mg/mL BSA). Initial rates (<20% reaction) of enzyme-catalyzed hydrolysis of pNP-GlcNAc and pNP-GalNAc at substrate concentrations spanning 0.2-5.0 Km were determined on a Synergy H1 plate reader (BioTek) at 405 nm in a 96 well plate with 200 µL of solution per assay. Absorbance values were converted to concentrations using the respective extinction coefficients of para-nitrophenol in the buffer. Michaelis-Menten parameters (Vmax and Km) were extracted from these data sets by non-linear fitting to the Michaelis-Menten equation. Kinetic parameters using pNP-SGlcNAc were determined on a Synergy H1 plate reader (BioTek). Michaelis-Menten parameters (Vmax and Km) were extracted from these data by non-linear fitting to the Michaelis-Menten equation using the program Grafit 7.0. Km and Kcat values were obtained by measuring rates in a series of cells at a range of substrate concentrations. Absorbance values were converted to concentrations using the respective extinction coefficients of pNPS in the used buffer. pNP-SGlcNAc was synthesized following a published approach.[10]

The pH profile of SpHex E314A was determined using pNP-GlcNAc as substrate by performing a depletion assay at -0.1*Km (2.0 µM pNP-GlcNAc) at 37°C. The buffers contained 100 mM NaCl and 2 mg/mL BSA; pH 3-6.5: 25/25 mM citrate/phosphate; pH 7-8: 50 mM HEPES; pH 9-10: 50mM CHES. Release of pNP was detected at 405 nm (pH 6-10) and 348 nm (pH 3-6). A total of 2 or 3 runs were performed at each pH value: curves shown are representative. Exact pH values studied were: 3.3, 4.1,5.2, 6.2, 6.9, 7.9, and 8.7.

Thioglycoligation reactions: acceptor screening

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SUPPORTING INFORMATION
Thioglycoligase reactions were performed at pH 7.0 in HEPES buffer (25 mM HEPES, 100 mM NaCl, 2 mg/mL BSA, 10 mM DTT). The respective thio sugar acceptor was dissolved in DMSO and diluted with buffer resulting in a 5 mM acceptor solution (5% DMSO). GlcNAc oxazoline (GlcNAc-oxa), which was synthesized following a procedure of Andre-Miral et al [3], was used as donor for the initial screening, and pNP-GlcNAc or pNP-GalNAc was used for all further reactions on larger scales. The final assay concentrations were 30 mM donor, 5 mM thiol acceptor and 0.5 mg/mL SpHex E314A. All reactions for the acceptor screening were performed on a 100 µL scale at 37°C and monitored by TLC (5 EIOac: 2 MeOH: 1 H2O) and ESI-MS. TLC spots were visualized via UV and subsequently stained with an anisaldehyde stain. The formation of the desired S-glycosidic bonds was further confirmed by TLC using a thiol-specific stain (1 mM DTNB in 0.1 M NaOAc), only staining compounds with free thiols.[5] All thio sugars (see Table 2) were synthesized according to literature procedures.[5]

**Glcnac coupling to phenols**

The efficiency of the ligase reaction of SpHex E314A was evaluated using different phenols of varying pKa values as model acceptors; 2,4-Dinitrophenol (pKa 4.1), 4-nitrophenol (pKa 7.2), 2-chlorophenol (pKa 8.6) and phenol (pKa 10.0) were chosen for this purpose. All reactions were performed at pH 8.0 in HEPES buffer (25 mM HEPES, 100 mM NaCl, 2 mg/mL BSA) and GlcNAc-oxa was used as activated donor substrate. Final concentrations in the assay were 5 mM GlcNAc-oxa, 0.5 mg/mL SpHex E314A and 20 mM of the respective phenol, except 2-chlorophenol which was used at 7 mM due to its limited solubility. Reactions were performed at 37°C and product formation was monitored by TLC (qualitative assay). The synthesis rate of SpHex E314A was determined using pNP as acceptor, following the decrease in absorption as a function of GlcNAc ligation to pNP. Reagent concentrations were chosen to be 2 mM GlcNAc-oxa donor and 40 µM pNP acceptor. Synthesis rates were determined at pH 7.0 (HEPES), pH 8.0 (HEPES), and pH 9.0 (CHES); molar extinction coefficients for the phenols were determined using identical buffers.

**Peptide synthesis**

Synuclein model peptides (pentamer) were synthesized covering the in vivo GlcNAcylation sites T72 (AGTIA) and S87 (VVSGV).[6,7] Additionally, the same peptides were synthesized but with the GlcNAcylation sites replaced by cysteine (AGCIA / VVCGV), respectively, enabling S-GlcNAcylation by SpHex E314A.

Automated peptide synthesis was performed using an Intavis MultiPep system. Fmoc rink amide MBHA resin was used throughout the syntheses. Peptides were synthesized under standard automated Fmoc protocols: Using DMF as solvent, deblock for 5 min, couple for 2 x 10 min. The following Fmoc amino acids (NovaBiochem) were employed: Fmoc- Ala- OH, Fmoc- Cys(Trt)-OH, Fmoc- Gly- OH, Fmoc- His(Trt)-OH, Fmoc- Ile- OH, Fmoc- Ser(Bu)-OH, Fmoc- Thr(Bu)-OH, Fmoc- Val- OH. Excess amino acids (4 eq.), coupling reagent (HBTU, 4 eq.) and acide modifier (HOBT, 1 eq.) was employed for each cycle. NMM (4 eq.) was used as base in the coupling step, and deblock solution was prepared as a mixture of DMF/piperidine (4:2, v/v).

After the final deprotection, the cleavage of the peptides from the resin was performed periodically by adding a solution of TFA/phenol/water/TIPS (88:5:5:2) over 1 h. Crude peptides were precipitated from the TFA solution by 100-fold dilution into ice cold diethyl ether. The precipitate was washed 3 times with ice cold diethyl ether and dried. Crude peptides were dissolved in 50% MeCN and the filtered solution was subjected to HPLC purification using the same equipment and column as mentioned before. A linear gradient was applied (5-95% MeCN, 0.1% TFA, over 15 min) and the respective product fractions were confirmed by ESI-MS.

**Thioglycoligase of GlcNAc to synuclein model peptides and a Tau S400C variant**

The thioglycoligase to peptides and Tau (244-441) S400C was performed using a similar approach to that described for the sugar acceptor screen, again using HEPES buffer. Tau (244-441) wt contains 2 native cysteines C301 and C322 which were mutated to serines; thus the version of the tau protein (Tau S400C) used was the triple mutant C301S C322S T400C. GlcNAcylation of the synuclein cysteine peptide variants was performed in the presence of 10 mM DTT, preventing disulfide formation, 10 mM pNP-GlcNAc and 1 mg/mL of the respective peptide. Reactions were initiated by the addition of SpHex E314A (0.1 mg/mL final concentration). For the S-GlcNAcylation of Tau (244-441) S400C all experiments were performed in bicarbonate buffer (20 mM, pH 7.0) using final concentrations of 160 µM Tau S400C and 6.5 mM pNP-GlcNAc. All reactions were incubated at 37°C for 3 h prior to product analysis.

Mass spectrometry

The synuclein peptide reactions were analyzed on a Bruker AutoFlex MALDI TOF MS using a Super DHB matrix (dissolved in 0.1% TFA), whereby 1 µL of a 1:1 mixture of matrix and reaction solution was spotted and dried on a MALDI sample plate. HPLC analyses of the reactions were performed on the above-mentioned instrument and column using the following gradient: 0.3 min 95% H2O, 3-5 min to 75% H2O, 5-20 min to 5% H2O. Reaction yields were determined by HPLC, comparing the integrals of S-GlcNAcylated peptides to those of the peptide starting material.
S-GlcNAcylation reactions with Tau (244-441) S400C were analyzed on the intact protein by ESI-TOF MS on a Waters Xevo G2-S qTOF MS. Samples were diluted in 0.1% formic acid resulting a final concentration of 3 µg/mL and subjected to a Waters nanoACQUITY UPLC, equipped with a Zorbax 300SB-C8 column (Agilent): aliquots of 5 µL were subjected to MS analysis. Data were analyzed with a Waters MassLynx V4.1 mass spectrometer and a MaxEnt1 algorithm was used for deconvolution of the protein mass from the multiply charged species.

Titration of free thiols on tau
The selective GlcNAcylation of Cys 400 of Tau S400C was confirmed by titrating the remaining free thiols of Tau S400C, using a modified version of the method of Rienert et al.[8] GlcNAcylation experiments were performed as described above. Afterwards, tau was purified from DTT and other small molecule impurities using an Amicon Ultra centrifugal filters with a MWCO of 3 kDa (Merck) and repeatedly washing with 0.1 M NH₄HCO₃. The retentate was freeze-dried, afterwards dissolved in 100 µL ddH₂O and the protein concentration was determined using the Bradford reagent (Thermo scientific), using 10µL of sample. The samples were freeze-dried again, dissolved in 100 µL sodium phosphate buffer (100 mM NaH₂PO₄, 0.2 mM EDTA, pH 7.0) containing 6 M guanidinium-Cl and incubated at rt for 10 min. The 100 µL were split in aliquots of 50, 30, 10 and 5 µL, respectively, and diluted to a final volume of 100 µL using the same buffer. Two µL of the thiol titration reagent was added, consisting of phosphate buffer, 6 M guanidinium-Cl and 40 mM DTNB; the mixture was incubated in a Synergy H1 plate reader (BioTek) at 25°C and release of 2-nitro-5-thiobenzoate was monitored at 412 nm. Negative controls were measured mixing the mentioned buffer with 2 µL titration reagent monitoring eventual spontaneous hydrolysis of the DTNB reagent. The maximal absorption values of the different dilutions were plotted against the protein concentration. The slope of the linear regression is a measure of the free thiols present content and the values of the slopes were corrected with the protein concentration (=correction factor) to take into account varying protein concentrations due to different protein recovery from the Amicon purifications.

The yield of S-GlcNAcylation was calculated directly comparing the corrected slopes of the SpHex GlcNAcylated tau to untreated tau. Due to the reduced shelf life of the DTNB titration reagent, experiments with BSA (bearing one free thiol) were conducted regularly to validate the method.
2) Results and Discussion

1. **NMR Data**

1.1. GlcNAc-(β-1,4)-4-S-GlcNAc-β-pNP

**H NMR** (400 MHz, D$_2$O): δ 2.02 (3H, s, HAc), 2.06 (3H, s, HAc'), 3.03 (1H, t, J 10.6 Hz, H4), 3.47-3.49 (2H, m, H4', H5'), 3.57-3.61 (1H, m, H3'), 3.72-3.77 (3H, m, H3, H6 R/S', H2'), 3.89-3.92 (1H, m, H5), 3.94-3.97 (2H, m, H6 R/S, H6 R/S'), 4.03 (1H, dd, J 6.2 Hz, H2), 4.08-4.11 (1H, m, H6 R/S), 4.77 (1H, d, J 10.2 Hz, H1'), 5.30 (1H, d, J 8.5 Hz, H1), 7.18 (2H, d, J 9.3 Hz, HAr a), 8.25 (2H, d, J 9.3 Hz, HAr b); **C NMR** (100 MHz, D$_2$O): δ 22.06 (CAc), 22.10 (CAc'), 47.8 (C4), 54.9 (C2'), 56.5 (C2), 60.7 (C6'), 61.1 (C6), 69.6 (C4'), 70.9 (C3), 74.8 (C3'), 76.8 (C5), 79.8 (C5'), 83.8 (C1'), 98.3 (C1), 116.5 (CAr a), 126.1 (CAr b)

**HRMS**: calcd for C$_{22}$H$_{31}$N$_3$O$_{12}$SNa: 584.1526; found: 584.1520
1.2. GlcNAc-(β-1,3)-3-S-GlcNAc-β-pNP 2

**H NMR** (400 MHz, D₂O): δ 2.02 (6H, s, HAc), 2.03 (3H, s, HAc'), 3.08 (1H, t, J 7.1 Hz, H3), 3.47-3.49 (2H, m, H4', H5'), 3.60-3.69 (2H, m, H4, H3'), 3.69-3.74 (1H, m, H2'), 3.71-3.78 (1H, m, H5), 3.80-3.84 (2H, m, H6 R/S', H6 R/S'), 3.91-3.99 (2H, m, H6 R/S, H6 R/S) 4.13 (1H, t, J 6.7 Hz, H2), 4.78 (1H, d, J 11.4 Hz, H1'), 5.32 (1H, d, J 8.3 Hz, H1), 7.19 (2H, d, J 9.3 Hz, HAr a), 8.26 (2H, d, J 9.3 Hz, HAr b); **13C NMR** (100 MHz, D₂O): δ 22.0 (C Ac), 22.1 (C Ac'), 52.0 (C3), 54.0 (C2), 54.9 (C2'), 60.7 (C6, C6'), 67.2 (C4), 69.6 (C4'), 74.6 (C3'), 78.6 (C5), 79.9 (C5'), 84.0 (C1'), 99.7 (C1), 116.5 (C Ar a), 126.1 (C Ar b).

**HRMS**: calcd for C₂₂H₃₁N₃O₁₂SNa: 584.1526; found: 584.1522
1.3. GlcNAc-β-1,6-6-S-GlcNAc-β-pNP 3

$^1$H NMR (400 MHz, D$_2$O): $\delta$ 1.96 (3H, s, HAc), 2.03 (3H, s, HAc'), 2.83-2.89 (1H, m, H6 R/S), 3.35 (1H, dd, J 5.4 Hz, H6 R/S), 3.41-3.45 (1H, m, H5'), 3.45-3.50 (1H, m, H4'), 3.45-3.54 (2H, m, H4, H3'), 3.64-3.69 (1H, m, H3), 3.68-3.72 (1H, m, H6 R/S'), 3.78 (1H, t, H2'), 3.86-3.90 (1H, m, H5), 3.89-3.93 (1H, m, H6 R/S'), 4.06 (1H, t, J 6.3 Hz, H2), 4.66 (1H, d, J 10.4 Hz, H1'), 5.31 (1H, d, J 8.4 Hz, H1), 7.25 (2H, d, J 9.3 Hz, HAr a), 8.27 (2H, d, J 9.3 Hz, HAr b);

$^{13}$C NMR (100 MHz, D$_2$O): $\delta$ 22.07 (CAc, CAc'), 32.2 (C6), 54.8 (C2'), 55.3 (C2), 60.9 (C6'), 68.8 (C4'), 72.8 (C4), 73.3 (C3), 75.1 (C3'), 75.3 (C5), 79.8 (C5'), 84.9 (C1'), 98.3 (C1), 116.6 (CAr a), 126.1 (CAr b)

HRMS: calcd for C$_{22}$H$_{31}$N$_3$O$_{12}$SNa: 584.1526; found: 584.1522
1.4. GalNAc-(β-1,4)-4-S-GlcNAc-β-pNP 4

$^1$H NMR (400 MHz, D$_2$O): δ 2.01 (3H, s, HAc), 2.06 (3H, s, HAc'), 3.02 (1H, t, J 10.5 Hz, H4), 3.70-3.80 (1H, m, H4'), 3.71-3.76 (1H, m, H5'), 3.45-3.50 (1H, m, H4'), 3.72-3.78 (1H, m, H3'), 3.90-3.94 (1H, m, H5'), 3.90-3.97 (1H, m, H2'), 3.91-3.98 (2H, m, H6 R/S, H6 R/S'), 3.96-4.01 (1H, m, H3), 4.03 (1H, t, J 8.6 Hz, H2), 4.09-4.12 (2H, m, H6 R/S, H6 R/S'), 4.70 (1H, d, J 10.4 Hz, H1'), 5.30 (1H, d, J 8.4 Hz, H1), 7.18 (2H, d, J 9.3 Hz, Har a), 8.26 (2H, d, J 9.3 Hz, Har b); $^{13}$C NMR (100 MHz, D$_2$O): δ 22.0 (CAc), 22.1 (CAc'), 47.9 (C4), 51.4 (C2'), 56.4 (C2), 61.2 (C6, C6'), 70.1 (C3, C4'), 71.7 (C3'), 76.8 (C5), 79.0 (C5'), 84.2 (C1'), 98.3 (C1), 116.4 (Car a), 126.1 (Car b)

HRMS: calcld for C$_{22}$H$_{31}$N$_{3}$O$_{12}$SNa: 584.1526; found: 584.1519
1.5. GlcNAc-β-cysteine ethyl ester

$^1$H NMR (400 MHz, D$_2$O): δ 1.41 (3H, tr, J 7.1 Hz, H10), 2.15 (3H, s, HAc), 3.24 (1H, dd, J 7.8 Hz, H7a), 3.58-3.65 (3H, m, H4, H5, H7b), 3.64-3.70 (1H, m, H3), 3.86 (1H, dd, J 5.8 Hz, H6a), 3.97 (1H, t, J 10.2 Hz, H2), 4.03 (1H, d, J 12.2 Hz, H6b), 4.40-4.56 (2H, q, J 7.1 Hz, H9), 4.51 (1H, dd, J 3.8 Hz, H8), 4.72 (1H, d, J 10.2 Hz, H1); $^{13}$C NMR (100 MHz, D$_2$O): δ 13.1 (C10), 22.1 (CAc), 29.3 (C7a, C7b), 53.2 (C8), 53.7 (C2), 60.6 (C6, C6'), 63.9 (C9), 69.5 (C4), 74.7 (C3), 80.1 (C5), 83.1 (C1)

HRMS: calcd for C$_{13}$H$_{24}$N$_2$O$_7$SH: 353.1382; found: 353.1381
2. Figures

Scheme S1. (a) Wild type “Koshland-type” glycoside hydrolase mechanism. (b) nucleophile mutant acting as a glycosynthase using an α-glycosyl fluoride as donor. (c) acid/base mutant acting as a thioglycoligase using a β-donor with a good leaving group (LG).

Figure S1. pH profile of SpHex E314A using pNP-GlcNAc as substrate. The enzyme rapidly loses activity at pH 3.
Figure S2. ESI-MS analyses confirming the thioglycoligation products described in Figure S1. The thiosugar acceptor is shown on top of each product mass spectrum.

Figure S3. TLC screen of a thioglycoligation screen using SpHex E314A and a range of thiosugar acceptors. Structures on the top of each depict the thiosugar acceptor. “SpHex E314A” refers to the thioglycoligation reaction; the “Blank reaction” was performed in the absence of the enzyme. TLC spots were visualized by UV, the ligation donor GlcNAc-oxa is not UV-active and thus not visualized within this figure. The TLC’s shown were performed after ~15 min reaction time and thus do not depict full conversion of the thiosugar acceptor. Mobile phase: EtOAc:MeOH:H₂O (7:5:2)
**Figure S4.** TLC analysis of selected thioglycoligations, visualized with a p-anisaldehyde stain (left) and a DTNB stain (right) selectively staining free thiols; thioglycoligations using 4-S-GlcNAc pNP (A), 6-S-Glc pNP (B) and cysteine ethyl ester (C) as acceptors. "SpHex E314A" refers to the thioglycoligation reaction and pNP-GlcNAc was the used donor. Cysteine (ethyl ester) was the thioacceptor.

**Figure S5.** Synthesis and re-hydrolysis of pNP-GlcNAc by SpHex E314A at different pH values, photometrically detected at 405 nm. The linear decrease in absorption within the first minutes of the reaction was used to calculate the synthesis rate. The hydrolysis process started earlier at higher pH values.
Figure S6. Thioglycoligation of cysteine ethyl ester using SpHex E314A and pNP-GlcNAc as donor. (A) TLC analysis of the reaction, whereby "SpHex E314A" refers to the thioglycoligation reaction, pNP-GlcNAc is the donor and cysteine (ethyl ester) the thio acceptor. (B) MS confirmation of the thioglycoligation product; TLC spots were visualized using a p-anisaldehyde stain.

Figure S7. Blank reactions of the GlcNAcylation of Tau S400C using different donors: (a) GlcNAc oxazoline, (b) GlcNAc, (c) pNPGlcNAc.
Figure S8. Titration of free thiols of different concentrations of GlcNAcylated Tau S400C by SpHex E314A, compared to untreated Tau S400C. The slope of the trendline is a measure of the free thiols present in the solutions. A correction factor was calculated based on the protein concentration of the respective sample and the yield of S-GlcNAcylation was determined by directly comparing the corrected values of untreated Tau S400C to SpHex GlcNAcylated Tau S400C (table in the figure).
Figure S9. HPLC chromatogram of S-GlcNAcylation reactions of the α-synuclein model peptides AGCIA (A) and VVCIA (B). The pure peptides eluted at 12.1 min (AGCIA) and 12.6 min (VVCIA) and the synthesized glycopeptides eluted at 10.9 min and 11.4 min.
3. **Kinetics, Michaelis-Menten plots**

**pNP-GlcNAc, SpHex wt:** pH 5.0 (left), pH 7.0 (right)

- **pKP-GlcNAc, SpHex E314A:** pH 5.0 (left), pH 7.0 (right)

**pNPS-GlcNAc, pH 7.0:** SpHex wt (left), SpHex E314A (right)

| Parameter  | Value  | Std. Error |
|------------|--------|------------|
| $V_{\text{max}}$ | 120.10 | 7.84 |
| $K_m$      | 0.32   | 0.07       |

| Parameter  | Value  | Std. Error |
|------------|--------|------------|
| $V_{\text{max}}$ | 208.90 | 4.46 |
| $K_m$      | 0.15   | 0.01       |
4. Depletion assay raw data

**pH 3.1**

Initial Abs = 6.932e-02 ± 1.37e-05 AU
Initial Rate = 3.11e-03 ± 1.54e-07 Alphsec
Rate constant, k = 2.17e-03 ± 1.38e-06 Alphsec
Kcat/Km = 1.43e+03 ± 7.44e+02 M⁻¹sec⁻¹

**pH 4.0**

Initial Abs = 7.520e-02 ± 3.13e-05 AU
Initial Rate = 7.03e-03 ± 1.60e-07 Alphsec
Rate constant, k = 5.03e-03 ± 8.3e-06 Alphsec
Kcat/Km = 1.17e+03 ± 1.38e+02 M⁻¹sec⁻¹

**pH 5.0**

Initial Abs = 6.130e-02 ± 2.56e-05 AU
Initial Rate = 4.04e-03 ± 0.04 Alphsec
Rate constant, k = 1.14e-03 ± 1.48e-05 Alphsec
Kcat/Km = 1.60e+03 ± 1.56e+02 M⁻¹sec⁻¹

**pH 6.0**

Initial Abs = 3.653e-03 ± 4.4e-05 AU
Initial Rate = 5.00e-04 ± 4.11e-07 Alphsec
Rate constant, k = 3.00e-04 ± 1.21e-05 Alphsec
Kcat/Km = 1.34e+03 ± 1.34e+02 M⁻¹sec⁻¹

**pH 7.0**

Initial Abs = 2.653e-03 ± 1.25e-05 AU
Initial Rate = 8.63e-04 ± 9.0e-06 Alphsec
Rate constant, k = 5.43e-04 ± 1.60e-05 Alphsec
Kcat/Km = 1.43e+03 ± 1.43e+02 M⁻¹sec⁻¹

**pH 8.0**

Initial Abs = 1.50e-03 ± 1.50e-05 AU
Initial Rate = 1.43e-04 ± 1.50e-07 Alphsec
Rate constant, k = 3.14e-05 ± 9.46e-07 Alphsec
Kcat/Km = 1.74e+03 ± 2.15e+02 M⁻¹sec⁻¹

**pH 9.0**

Initial Abs = 3.74e-04 ± 3.74e-05 AU
Initial Rate = 1.01e-04 ± 1.34e-07 Alphsec
Rate constant, k = 5.34e-06 ± 6.16e-07 Alphsec
Kcat/Km = 6.06e+03 ± 6.06e+02 M⁻¹sec⁻¹
SUPPORTING INFORMATION

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Author Contributions

G. Tegl and J. Hanson carried out most of the experimental work and wrote a first draft of the manuscript. D. Kwan and A. Gonzalez-Santana carried out preliminary cloning, mutagenesis and characterization work and edited the manuscript. H. Chen synthesized the set of thiosugars. S. Withers conceived and directed the project, and edited the manuscript.