Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2022

Supplementary Information

Novel triple mutant of an extremophilic glycosyl hydrolase allows the rapid synthesis of thioglycosides

Lauriane Pillet,[a] David Lim,[a] Nourah Almulhim,[b] Ana I. Benítez-Mateos,[a] and Francesca Paradisi *[a,b]

[a] *Department of Chemistry, Biochemistry, and Pharmaceutical Sciences, University of Bern, Freiestrasse 3, CH-3012 Bern, Switzerland*

[b] *School of Chemistry, University of Nottingham, University Park, NG7 2RD, Nottingham, UK*

** E-mail: francesca.paradisi@unibe.ch*

Table of contents

1. Materials and methods

1.1. Reagents

All components required to perform PCR reactions were purchased from New England Biolabs (NEB), except the QuickChange Lightning multi site-directed mutagenesis kit that was purchased from Agilent. Primers were synthetised by the Microsynth company. GeneJET plasmid miniprep kit was purchased from ThermoFisher Scientific. All chemical reagents were acquired from Sigma Aldrich (Merck KGaA, Darmstadt) unless otherwise specified. The HisTrapFF crude® column used for protein purification was purchased from Cytiva. 4-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) used for activity assays was purchased from Carbosynth. All commercial chemicals were used without further purification. *p*-nitrophenyl-β-D-thioglucopyranoside (*p*NP-Glc), phenyl-β-D-glucopyranoside, *p*-toluyl-β-D-glucopyranoside and *p*-bromophenyl-β-D-thioglucopyranoside were synthetised in house according to literature procedure. The synthetic details are indicated in section **1.6: Synthesis of the thioglycoside standards**.

1.2. DNA preparation and site-directed mutagenesis

The different *Hor*GH1 variants used in this study were produced *via* the traditional quick-change PCR using the Q5® high-fidelity DNA polymerase (*Hor*GH1 E166A, *Hor*GH1 M299R/E166A), *via* the Q5® site-directed mutagenesis kit (*Hor*GH1 E354G, *Hor*GH1 M299R/E354G) or *via* the QuickChange Lightning multi site-directed mutagenesis kit (*Hor*GH1 M299R, *Hor*GH1 E166A/E354G, *Hor*GH1 M299R/E166A/E354G). The pET45b plasmid hosting the ds-DNA of HorGH1 (GeneBank accession number WP_012636460)[1,2] and the HorGH1 M299R[1] variants were used as templates in the PCR reactions.

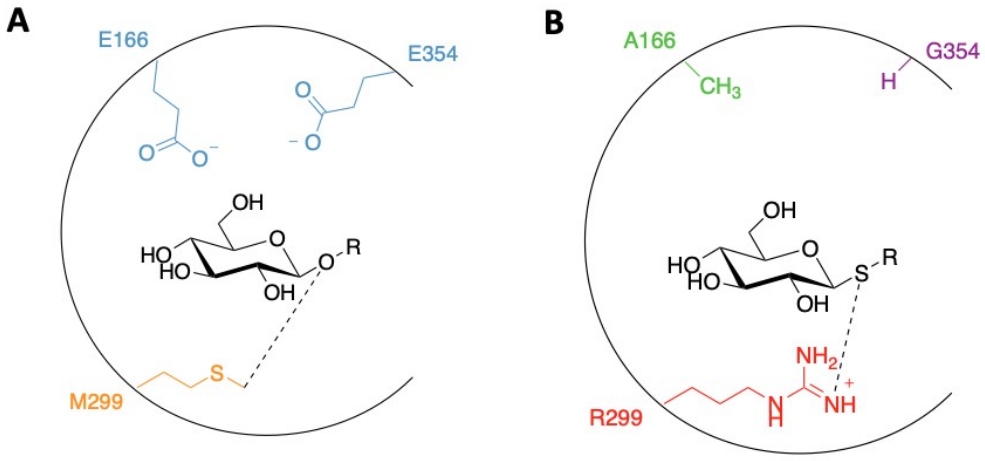
1.2.1. Primers design

Table S1: Sequences of the primer used to design the *Hor*GH1 variants.

Entry	Primer type	Variants created	Sequence
1	M299R	*Hor*GH1 M299R	5'-AATTATTACAGTAGAAGGGTCGTACGCCATAAG-3'
2	E166A forward (F_E166A)	*Hor*GH1 E166A, *Hor*GH1 M299R E166A	5'-GTGACTCATAACGCACCGTGGGTTGTGG-3'
3	E166A reverse (R_E166A)	*Hor*GH1 E166A, *Hor*GH1 M299R E166A	3'-CCACAACCCACGGTGCGTTATGAGTCAC-5'
4	E354G forward (F_E354G)	*Hor*GH1 E3454G, *Hor*GH1 M299R E354G	5'-CTTTGTACATTACTGGTAACGGTGCCGCGTT-3'
5	E354G reverse (R_E354G)	*Hor*GH1 E3454G, *Hor*GH1 M299R E354G	3'-GCTTGTCGGTGTATTCTTTGTTTACTCTGACC-5'
6	E166A	*Hor*GH1 E166A E3454G, *Hor*GH1 M299R E166A E3454G	5'-GGGTGACTCATAACGCACCGTGGGTTGTGG- 3'
7	E354G	*Hor*GH1 E166A E3454G, *Hor*GH1 M299R E166A E3454G	5'-CAAGCCTTTGTACATTACTGGAAACGGTGCCGCGT-3'

Once the PCR reactions were performed, produced DNA was used to transform by heat-shock at 42 °C the appropriate competent cells adapted to each PCR protocol. The GeneJET plasmid miniprep kit from ThermoFisher Scientific was used to extract the DNA for sequencing.

1.2.2. *Hor*GH1 variants mutations and sequences



C

MAHHHHHHVGTGSNDDDDKSPMAKIIFPEDFIWGAATSSYQIEGAFNEDGKGESIWDRFSHTPGKIENGDTGDIACDHY
HLYREDIELMKEIGIRSYRFSTSWPRILPEGKGRVNQKGLDFYKRLVDNLLKANIRPMITLYHWDLPQALQDKGGWTNRDT
AKYFAEYARLMFEEFNGLVDLWVTHNEPWVVAFEGHAFGNHAPGTKDFKTALQVAHHLLLSHGMAVDIFREEDLPGEIGI
TLNLTPAYPAGDSEKDVKAASLLDDYINAWFLSPVFKGSYPEELHHIYEQNLGAFTTQPGDMDIISRDIDFLGINYYSRMVVR
HKPGDNLFNAEVVKMEDRPSTEMGWEIYPQGLYDILVRVNKEYTDKPLYITENGAAFDDKLTEEGKIHDEKRINYLGDHFK
QAYKALKDGVPLRGYYVWSLMDNFEWAYGYSKRFGLIYVDYENGNRRFLKDSALWYREVIEKGQVEANGYPNWELVYTA
RLQVDKLAAALESGKETAAAKFERQHMDSSTSAA

D

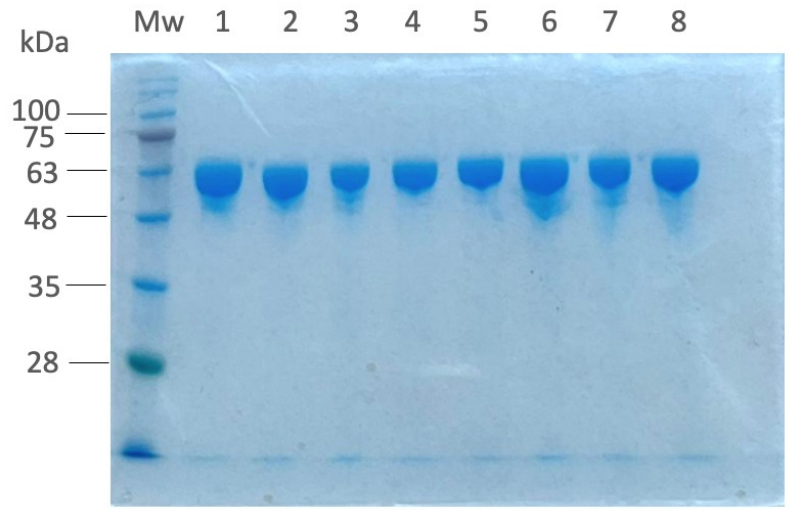
MAHHHHHHVGTGSNDDDDKSPMAKIIFPEDFIWGAATSSYQIEGAFNEDGKGESIWDRFSHTPGKIENGDTGDIACDHY
HLYREDIELMKEIGIRSYRFSTSWPRILPEGKGRVNQKGLDFYKRLVDNLLKANIRPMITLYHWDLPQALQDKGGWTNRDT
AKYFAEYARLMFEEFNGLVDLWVTHNAPWVVAFEGHAFGNHAPGTKDFKTALQVAHHLLLSHGMAVDIFREEDLPGEIGI
TLNLTPAYPAGDSEKDVKAASLLDDYINAWFLSPVFKGSYPEELHHIYEQNLGAFTTQPGDMDIISRDIDFLGINYYSRRVVR
HKPGDNLFNAEVVKMEDRPSTEMGWEIYPQGLYDILVRVNKEYTDKPLYITGNGAAFDDKLTEEGKIHDEKRINYLGDHFK
QAYKALKDGVPLRGYYVWSLMDNFEWAYGYSKRFGLIYVDYENGNRRFLKDSALWYREVIEKGQVEANGYPNWELVYTA
RLQVDKLAAALESGKETAAAKFERQHMDSSTSAA

Figure S1: Diagram of the catalytic site of **A**) wild type *Hor*GH1 **B**) *Hor*GH1 M299R/E166A/E354G variant. Protein sequences of wild type *Hor*GH1 (**C**) and *Hor*GH1 M299R/E166A/E354G (**D**). Wild type E166 and E354 glutamic acid residues are highlighted in blue and M299 methionine residue is highlighted in orange in the wild type *Hor*GH1. E166A and E354G mutations of glutamic acid residues in alanine and glycine residues are highlighted in green and violet, respectively, while M229R mutation is highlighted in red in the *Hor*GH1 M299R/E166A/E354G variant.

1.3. *Hor*GH1 variants expression, purification and quantification

The pET45b plasmids harboring the genes of the different *Hor*GH1 variants were transformed into chemically competent *E.coli* BL21(DE3) cells. Then, 1L flasks containing 300 mL of autoinduction media (10 g/L N-Z-amine, 5 g/L yeast extract, 50 mM KH_2PO_4, 50 mM K_2HPO_4, 25 mM $(NH_4)_2SO_4$) with 100 µg/mL ampicillin were inoculated with a single colony and incubated at 37 °C and 150 rpm for 24 hours. Cells (3 g) were harvested by centrifugation at 2500·*g*, 4 °C, 20 min and resuspended in 7.5 mL of loading buffer (50 mM HEPES, 150 mM NaCl, 10 mM imidazole, pH 7.5). The suspension was placed on ice and lysed by sonication at 40 % amplitude for 8 min, with pulses of 5 s ON, 10 s OFF. After centrifugation at 12,100·g, 4 °C, 45 min, the supernatant was filtered with a 0.45 µm filter. *Hor*GH1 variants were then purified by metal affinity chromatography (IMAC) using an AKTA^{TM} Start. Pure fractions were pooled and dialysed for 20 h at room temperature with dialysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl) with one buffer exchange after 2h. Purification of the enzyme was checked by analysing the different fractions with a 12 % SDS PAGE, by staining with Coomassie blue (**Figure S2**). The concentration of the purified enzymes was estimated by measuring the absorbance at 280 nm in the EPOCH2 (nanodrop Take3 plate) using an extinction coefficient (ε) of 106,230 $L \cdot mol^{-1} \cdot cm^{-1}$ (predicted using the ExPASy ProtParam tool[1,2]) and a molecular weight of 52,003.56 Da.[1,5] Low protein concentrations (< 1 mg/mL) were determined by Bradford assay by measuring the absorbance at 595 nm of a solution containing 5 µL of protein sample with 250 µL of Bradford reagent. A calibration curve was performed with the BSA protein. Typically, starting from 3 g of wet cell paste, it was possible to obtain 25 mg of pure protein (5 fractions of 5 mg/mL, 83 mg/L of culture).

A



B

Variant	Expression yield [mg/$L_{culture}$]
*Hor*GH1	114.5
*Hor*GH1 E166A	81.1
*Hor*GH1 E354G	158.1
*Hor*GH1 E166A E354G	80.7
*Hor*GH1 M299R	34.1
*Hor*GH1 M299R E166A	82.1
*Hor*GH1 M299R E354G	80.1
*Hor*GH1 M299R E166A E354G	96.7

Figure S2: (**A**) SDS-PAGE after the expression and purification of the different *Hor*GH1 variants. Line 1: *Hor*GH1 Line 2: *Hor*GH1 E166A. Line 3: *Hor*GH1 E354G. Line 4: *Hor*GH1 E166A E354G. Line 5: *Hor*GH1 M299R. Line 6: *Hor*GH1 E166A M299R. Line 7: *Hor*GH1 E354G M299R. Line 8: *Hor*GH1 E166A E354G M299R. Mw: prestained protein ladder V from Geneaid. All protein samples were loaded in the SDS-PAGE at similar concentration. (**B**) Expression yields of the different *Hor*GH1 variants.

1.4. Activity assay

The activity of the *Hor*GH1 variants were measured in triplicates by monitoring the change in absorbance at 420 nm of *p*-nitrophenol (*p*NP) and *p*-nitrothiophenol (*p*NT). The molar extinction coefficient (ε) for the products were determined by obtaining a calibration curve of absorbance as a function of concentration (**Figure S3**) and was calculated according to Equation (1):

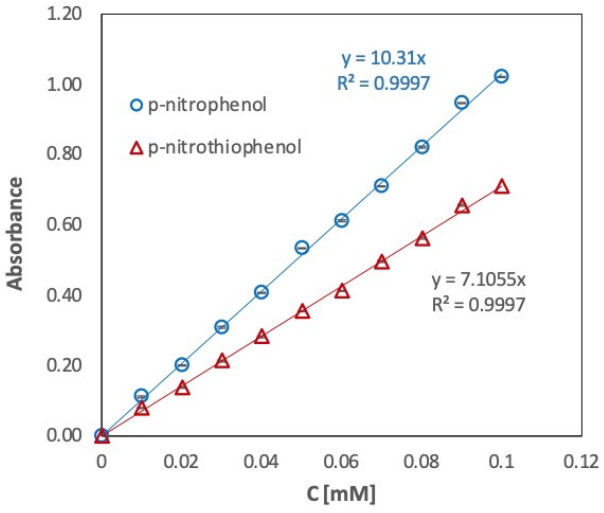
$$\varepsilon\left[L \cdot mol^{-1} \cdot cm^{-1}\right] = \frac{A}{l\,[cm] \cdot c[mol \cdot L^{-1}]} \quad (1)$$

With A the absorbance, l the pathlength and c the concentration. Activity assays were conducted in a 96-well plate at a total volume of 300 µL, at 25 °C and for 10 minutes. A typical reaction mixture contained 290 µL of 4-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc, 10 mM) or 4-nitrophenyl-β-D-thioglucopyranoside (*p*NT-Glc, 10 mM, concentration of DMSO controlled at 9 % across the assay) in 50 mM HEPES buffer pH 7.4 and 10 µL of the enzyme with appropriate dilution (approx. 60 µg/mL final concentration). The specific enzyme activity A_{HorGH1} [U/mg] was calculated according to Equation (2):

$$A_{HorGH1}\left[\frac{\mu mol}{min \cdot mg}\right] = \frac{S\,[\Delta A \cdot min^{-1}]}{\varepsilon\left[L \cdot mol^{-1} \cdot cm^{-1}\right] \cdot l\,[cm]} \cdot \frac{V_{tot}\,[\mu L] \cdot DF}{V_{enzyme}\,[\mu L] \cdot c_{enzyme}\left[\frac{mg}{mL}\right]} \cdot \frac{10^6\,[\mu mol \cdot mol^{-1}]}{10^3\,[mL \cdot L^{-1}]} \quad (2)$$

With S the slope of absorbance as a function of time, ε the molar extinction coefficient, l the pathlength, V_{tot} the total volume of the assay (300 µL), V_{enzyme} the volume of enzyme solution added in the assay (10 µL), DF the dilution factor of the enzyme solution and c_{enzyme} the concentration of the enzyme solution.

A **Calibration curves for *p*-nitrophenol and *p*-nitrothiophenol**



B

	p-nitrophenol (*p*NP)	*p*-nitrothiophenol (*p*NT)
ε [L·mol^{-1}·cm^{-1}]	10291.8	7125.5

Figure S3 A) Calibration curve of the absorbance as a function of p-nitrophenol (*p*NP) and p-nitrothiophenol (*p*NT) concentration **B)** Determination of the molar extinction coefficient ε [$L \cdot mol^{-1} \cdot cm^{-1}$].

1.5. Biotransformations

1.5.1. Screening of the thioglycosidic synthetic capability of the different *Hor*GH1 variants

Reactions were performed at 25 °C for 24 h in 50 mM bicarbonate buffer pH 9.4 containing 30 % DMSO, 1 mg/mL enzyme, 1 mM sugar donor, 20 mM β-mercaptoethanol (**Figure S4**) and 1, 2, 5 and 10 mM of *p*-nitrothiophenol (*p*NT) in a total reaction volume of 1 mL.

1.5.2. Screening of different reducing agents on enzyme performance

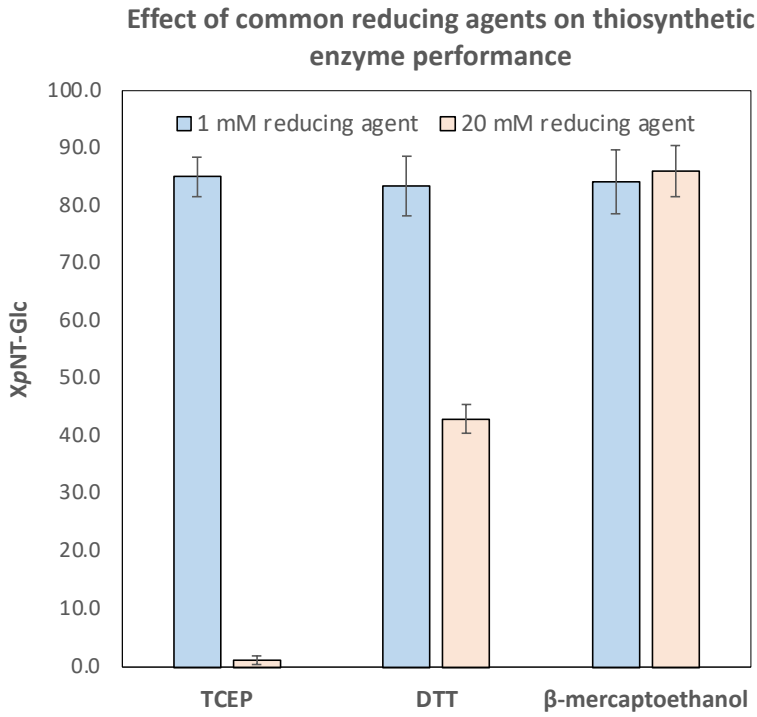
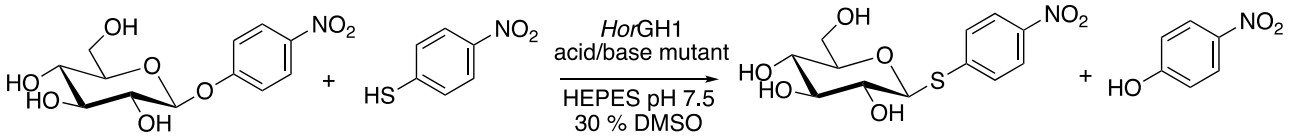


Figure S4: Screening of the effect of tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT) and β-mercaptoethanol reducing agents on *Hor*GH1 M299R/E166A/E354G thioglycosides synthetic performance.

1.5.3. *Hor*GH1 E166A and *Hor*GH1 M299R/E166A



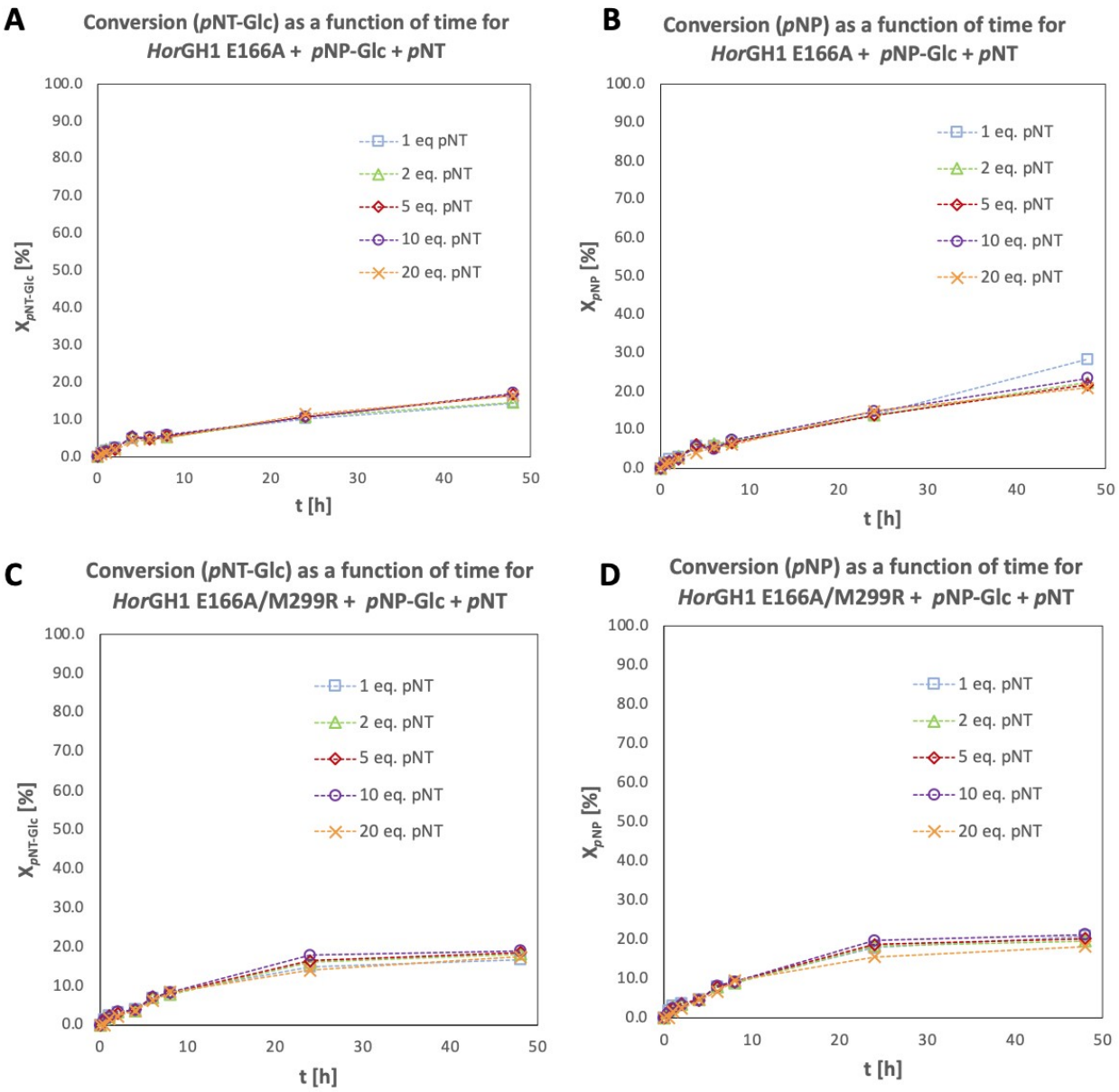
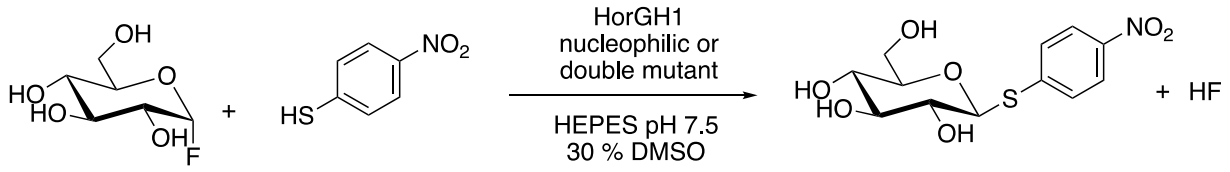


Figure S5: Conversion of **A)** 4-nitrophenyl-β-D-thioglucopyranoside (*p*NT-Glc, thioglycoside) and **B)** *p*-nitrophenol (pNP, leaving group) for reactions with the acid/base mutant E166A and conversion of **C)** 4-nitrophenyl-β-D-thioglucopyranoside (*p*NT-Glc, thioglycoside) and **D)** *p*-nitrophenol (*p*NP, leaving group) for reactions with the acid/base mutant M299R/E166A. Reactions were performed at 25 °C in 50 mM HEPES buffer pH 7.4 containing 30 % DMSO in the presence of 0.3 mg/mL enzyme, 1 mM sugar donor, 20 mM β-mercaptoethanol and an excess of *p*NT in a total reaction volume of 1 mL.

1.5.4. *Hor*GH1 E166A/E354G and *Hor*GH1 M299R/E166A/E354G



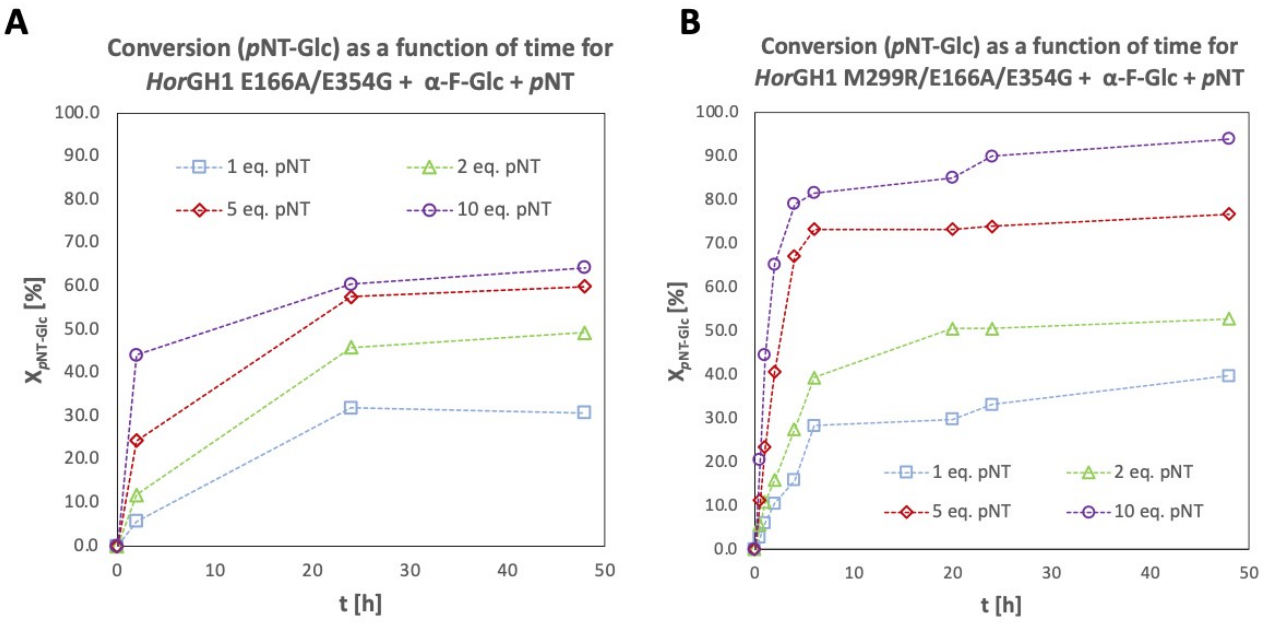


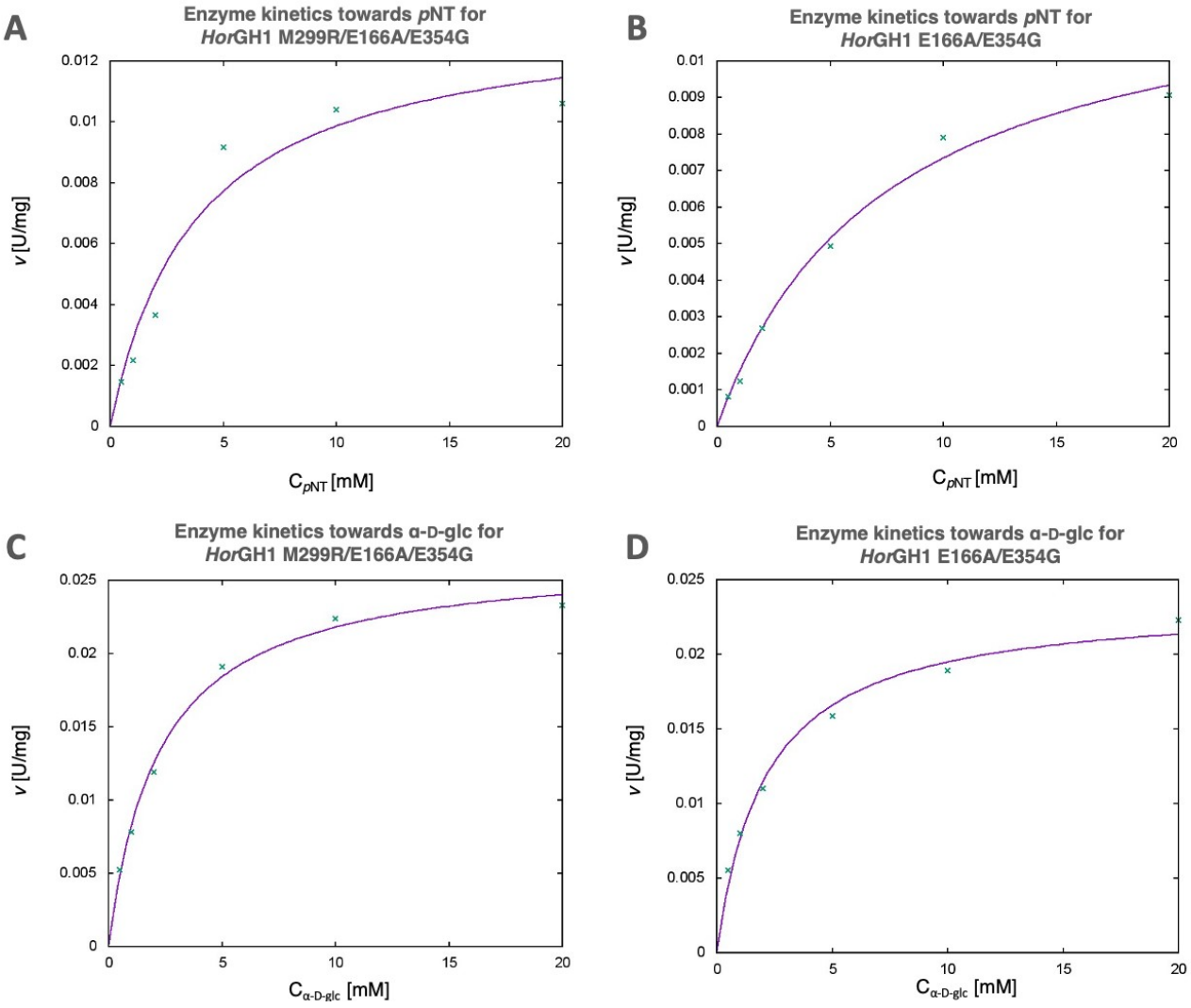
Figure S6: Conversion of 4-nitrophenyl-β-D-thioglucopyranoside (*p*NT-Glc, thioglycoside) for variants depleted of both catalytic residues. **A)** *Hor*GH1 E166A/E354G and **B)** *Hor*GH1 M299R/E166A/E354G. Reactions were performed at 25 °C in 50 mM HEPES buffer pH 7.4 containing 30 % DMSO in the presence of 0.3 mg/mL enzyme, 1 mM sugar donor, 20 mM β-mercaptoethanol and an excess of *p*NT in a total reaction volume of 1 mL.

1.5.5. Enzyme kinetics

Enzyme kinetics towards *p*NT were performed at 25 °C in 50 mM bicarbonate buffer pH 9.4 containing 30 % DMSO in the presence of 0.3 mg/mL enzyme, 1 mM sugar donor, 20 mM β-mercaptoethanol and 0.5 mM, 1 mM, 2 mM, 10 mM or 20 mM pNT in a total reaction volume of 1 mL. Enzyme kinetics towards α-D-glucopyranosyl fluoride were performed under the same conditions with 10 mM *p*NT and 0.5 mM, 1 mM, 2 mM, 5 mM, 10 mM or 20 mM α-D-glucopyranosyl fluoride. Reaction mixtures were analysed by HPLC at 15 min, 30 min, 1h, 2h, and 4h, and used to determine the activity [U/mg], which was calculated according to the following equation:

$$A_{HorGH1\ variants}\left[\frac{\mu mol}{min \cdot mL}\right] = \frac{S\left[mmol \cdot L^{-1} \cdot min^{-1}\right] \cdot 10^3\ [\mu mol \cdot mmol^{-1}]}{c_{enzyme}\left[mg \cdot mL^{-1}\right] \cdot 10^3\ [L \cdot mL^{-1}]}$$

With S [mM/min] the slope of conversion as a function of time and c_{enzyme} [mg/mL] the enzyme concentration in the biotransformation. K_M and V_{max} were then determined online.[3]



	***Hor*GH1 E166A E354G M299R**	***Hor*GH1 E166A E354G**
$K_{M,\ pNT}$ [mM]	3.82 ± 1.34	7.46 ± 1.27
$V_{max,\ pNT}$ [U/mg]	1.36 ± 0.17 · 10^{-2}	1.28± 0.09 · 10^{-2}
$K_{M,\ \alpha\text{-D-glc}}$	2.26 ± 0.23	2.12 ± 0.30
$V_{max,\ \alpha\text{-D-glc}}$ [U/mg]	2.67 ± 0.08 · 10^{-2}	2.36 ± 0.09 · 10^{-2}

Figure S7: Enzyme kinetics towards *p*NT for (**A**) *Hor*GH1 M299R/E166A/E354G and (**B**) *Hor*GH1 E166A/E354G and towards α-D-glucopyranosyl fluoride for (**C**) *Hor*GH1 M299R/E166A/E354G and (**D**) *Hor*GH1 E166A/E354G.

1.5.6. Confirmation of thioglycoside synthesis by ^{1}H NMR

The synthesis of the thioglycoside standards were performed as described in section **1.6. Synthesis of the thioglycoside standards** and the ^{1}H NMR spectra of the products were obtained using D_2O as the solvent (**Figure S7 A**). The enzymatic reaction was performed with the *Hor*GH1 M299R/E166A/E354G variant as described in section **1.5.1** in 50 mM HEPES buffer pH 7.4 containing 30 % deuterated DMSO with 10 mM of *p*NT. The water was then removed under reduced pressure, and 700 µL of d_6-DMSO was added (**Figure S7 B**). A ^{1}H NMR spectrum of the mixture was then obtained.

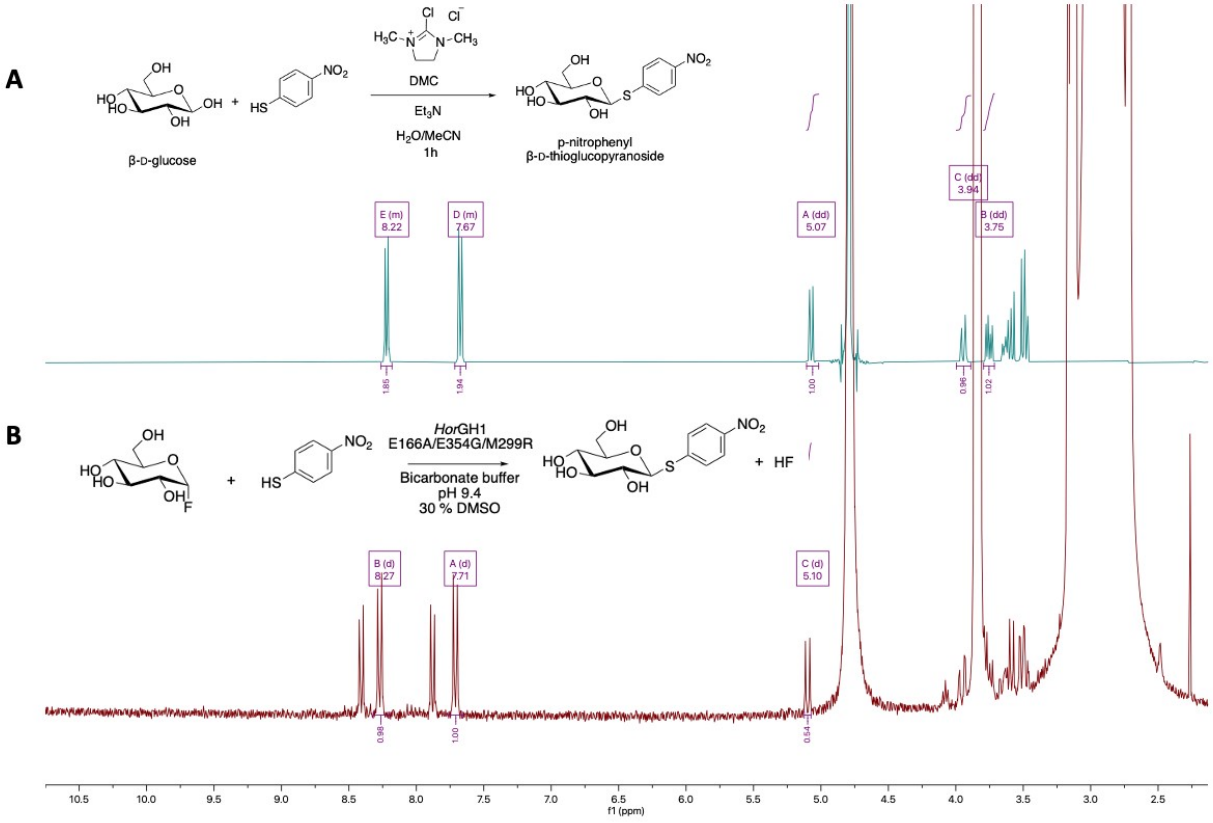


Figure S8: Overlaid ^{1}H NMR spectra of authentic *p*-nitrophenyl-β-D-thioglucopyranoside (*p*NT-Glc) (**A**) and the biocatalytic using our triple mutant *Hor*GH1 M299R/E166A/E354G (**B**). Chemical shifts for the anomeric and aromatic protons of the product are provided: δ_H (300 MHz, d_6-DMSO) 5.10 (1H, d, $J_{1,2}$ 9.6 Hz, H-1), 7.71 (1H, d, *J* 9.0 Hz, Ar-H), 8.22 (1H, d, *J* 9.0 Hz, Ar-H).

1.5.7. Activity of the different *Hor*GH1 variants with *p*NP-Glc and *p*NT-Glc

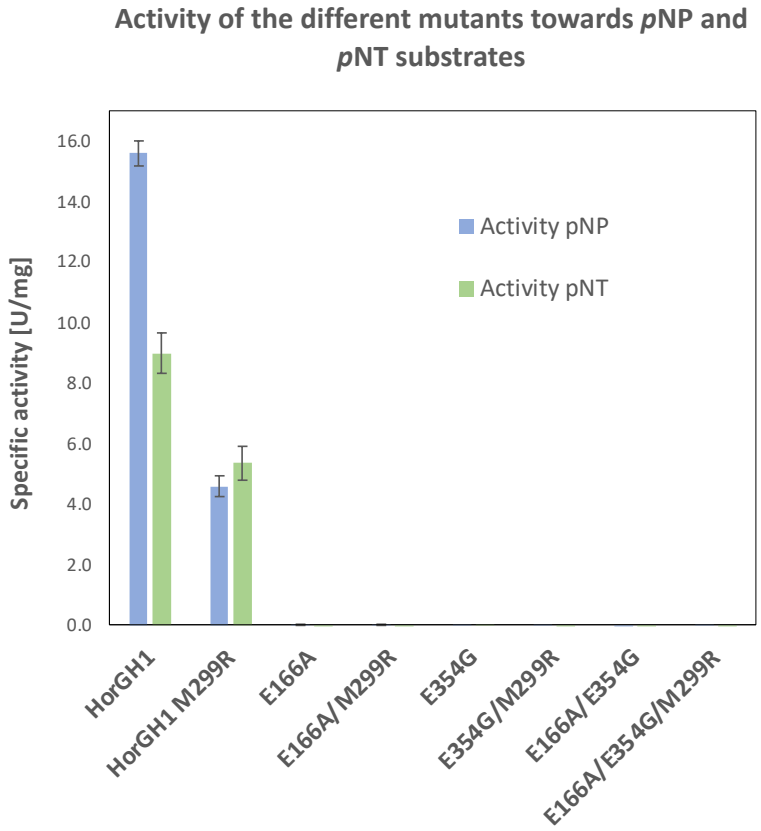


Figure S9: Activity of the different HorGH1 variants towards 4-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) and 4-nitrophenyl-β-D-thioglucopyranoside (*p*NT-Glc). All variants lacking either the catalytic acid/base or the nucleophilic residues had an activity of <0.01 U/mg towards *p*NP-Glc and no activity towards *p*NT-Glc. Among the mutants, *Hor*GH1 E166A and *Hor*GH1 M299R E166A displayed the highest activity (0.01 U/mg towards *p*NP-Glc), however this represents only 0.06 % of the wild type activity.

1.5.8. Screening of the thiols accepted by the *Hor*GH1 M299R/E166A/E354G variant

Reactions were performed with α-D-glucopyranosyl fluoride as the sugar donor and either *p*-nitrothiophenol, thiophenol, *p*-thiotoluene, and *p*-bromothiophenol as the acceptor. Reactions were performed at 25 °C in 70 % 50 mM bicarbonate buffer pH 9.4 containing 30% DMSO, 1 mg/mL enzyme, 1 mM sugar donor, 20 mM β-mercaptoethanol and 10 mM of *p*NT in a total reaction volume of 1 mL.

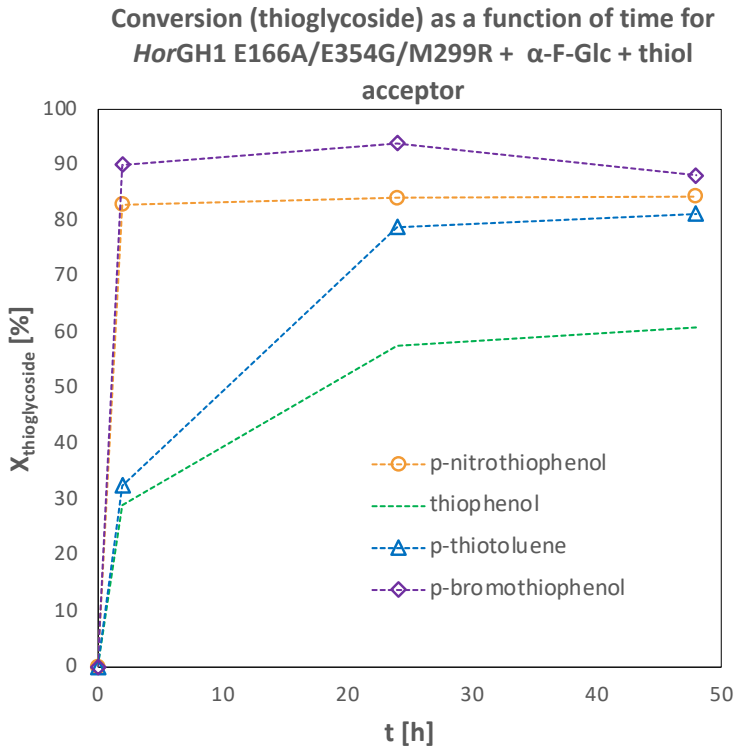


Figure S10: Conversion of the thioglycoside products resulting from the screening of thiols accepted by the *Hor*GH1 M299R/E166A/E354G variant. Reactions were performed at 25 °C in 50 mM bicarbonate buffer pH 9.4 containing 30 % DMSO in the presence of 1 mg/mL enzyme, 1 mM sugar donor and 10 equivalents excess of thiol acceptor in a total reaction volume of 1 mL.

1.5.7. Confirmation of thioglycoside synthesis by HRMS

Thioglycoside product	Formula	Calculated m/z	Obtained m/z
p-nitrophenyl-β-D-thioglucopyranoside (**3a**)	$C_{12}H_{15}O_7NS$	$(M + H^+)$ 318.0650	$(M + H^+)$ 318.0642
		$(M + Na^+)$ 340.0467	$(M + Na^+)$ 340.0461
phenyl-β-D-thioglucopyranoside (**3b**)	$C_{12}H_{16}O_5S$	$(M + H^+)$ 273.0800	$(M + H^+)$ 273.0791
		$(M + Na^+)$ 295.0611	$(M + Na^+)$ 295.0611
phenyl-β-D-thioglucopyranoside (**3c**)	$C_{13}H_{18}O_5S$	$(M + H^+)$ 287.0950	$(M + H^+)$ 287.0948
		$(M + Na^+)$ 309.0767	$(M + Na^+)$ 309.0767
p-bromophenyl-β-D-thioglucopyranoside (**3d**)	$C_{12}H_{15}BrO_5S$	$(M + H^+)$ 350.9900	$(M + H^+)$ 350.9896
		$(M + Na^+)$ 372.9718	$(M + Na^+)$ 372.9716
pyridinyl-β-D-thioglucopyranoside (**3f**)	$C_{11}H_{15}O_5S$	$(M + H^+)$ 274.0750	$(M + H^+)$ 274.0750
		$(M + Na^+)$ 296.0567	$(M + Na^+)$ 296.0570

Table S2: Calculated and obtained masses for the enzymatically synthetized thioglycosides

1.5.9. Screening of the effect of pH on thioglycoside conversion

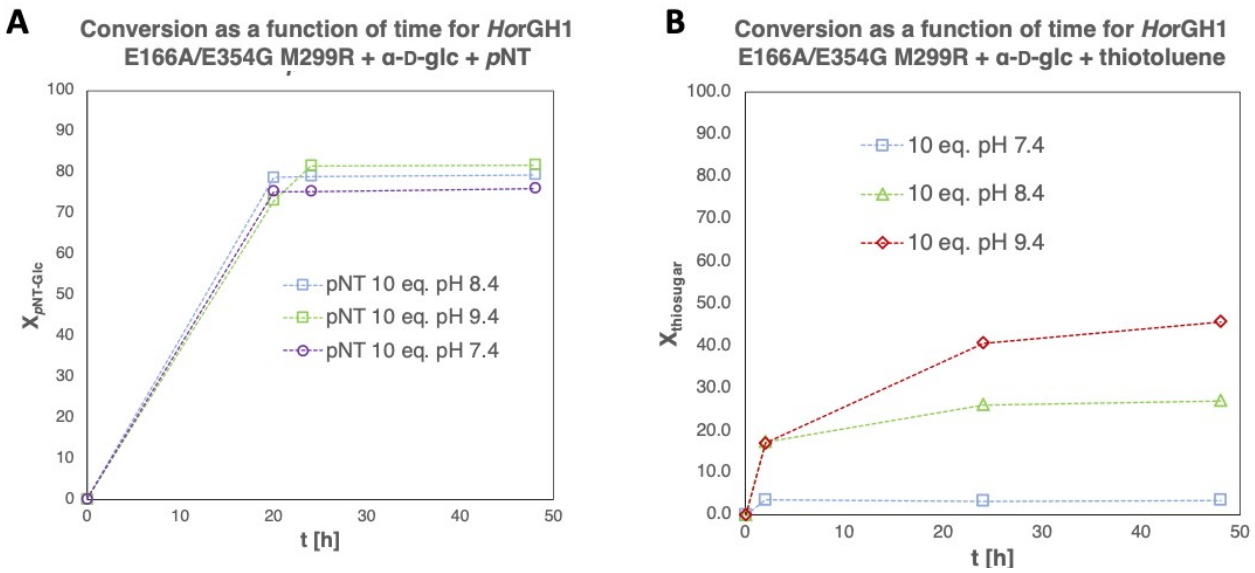


Figure S11: Effect of pH on the thioglycoside product conversion for the *Hor*GH1 M299R/E166A/E354G variant using 1 mM sugar donor and (**A**) 10 mM *p*-nitrothiophenol (*p*NT) (**B**) 10 mM thiotoluene as the thiol acceptor. Reactions were performed at 25 °C in 50 mM HEPES buffer pH 7.4, 50 mM phosphate buffer pH 8.4 or 50 mM bicarbonate buffer pH 9.4 containing 30 % DMSO.

1.5.10. HPLC analysis

Reactions were monitored by RP-HPLC for products **3a-d**. Conversions were determined based on product formation by HPLC (Dionex, UltiMate 3000 Thermo Fisher, Loughborough, UK) equipped with a C18 column 3.5 µm, 2.1Å, 150 mm (Waters, Elstree, UK). 2 µL of sample was injected and submitted to a gradient method of 5:95 to 95:5 [H_2O/ACN containing 0.1 % trifluoroacetic acid (TFA)] over 4 mins with a flow rate of 0.8 mL/min. Samples were detected using a UV detector at 330 nm. Calibration curves of the thioglycoside products (*p*-nitrophenyl-β-D-thioglucopyranoside, phenyl-β-D-glucopyranoside, *p*-toluyl-β-D-glucopyranoside and *p*-bromophenyl-β-D-thioglucopyranoside) were obtained using synthetic samples (**Figure S12**). 50 µL of reaction mixture was added to 225 µL of acetonitrile and 225 µL of 0.2 % aq. HCl in H2O. Samples were filtered through 0.45 µM PTFE filter prior to HPLC analysis. Conversions X [%] were calculated from HPLC analyses of the reaction mixture according to Equation (3):

$$X\,[\%] = \frac{A_{HPLC}\,[mAU \cdot min]}{Slope_{cal}\left[mAU \cdot min \cdot mM^{-1}\right] \cdot 1\,[mM]} \qquad (3)$$

With A_{HPLC} the peak area corresponding to the thioglycoside product (retention times exactly matched the calibration curve) and $Slope_{cal}$ the slope of the calibration curve. Examples of chromatograms can be found in **Figure S13**.

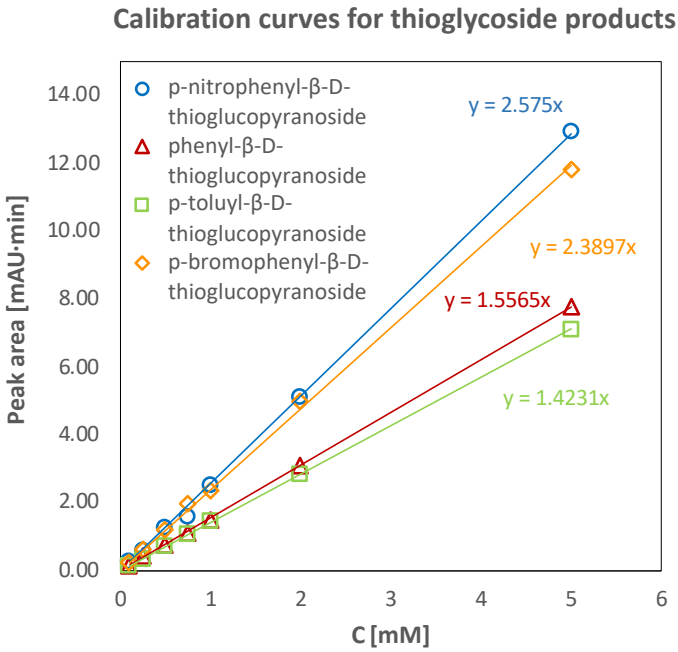


Figure S12: Calibration curves of the different thioglycosides using authentic samples of the product.

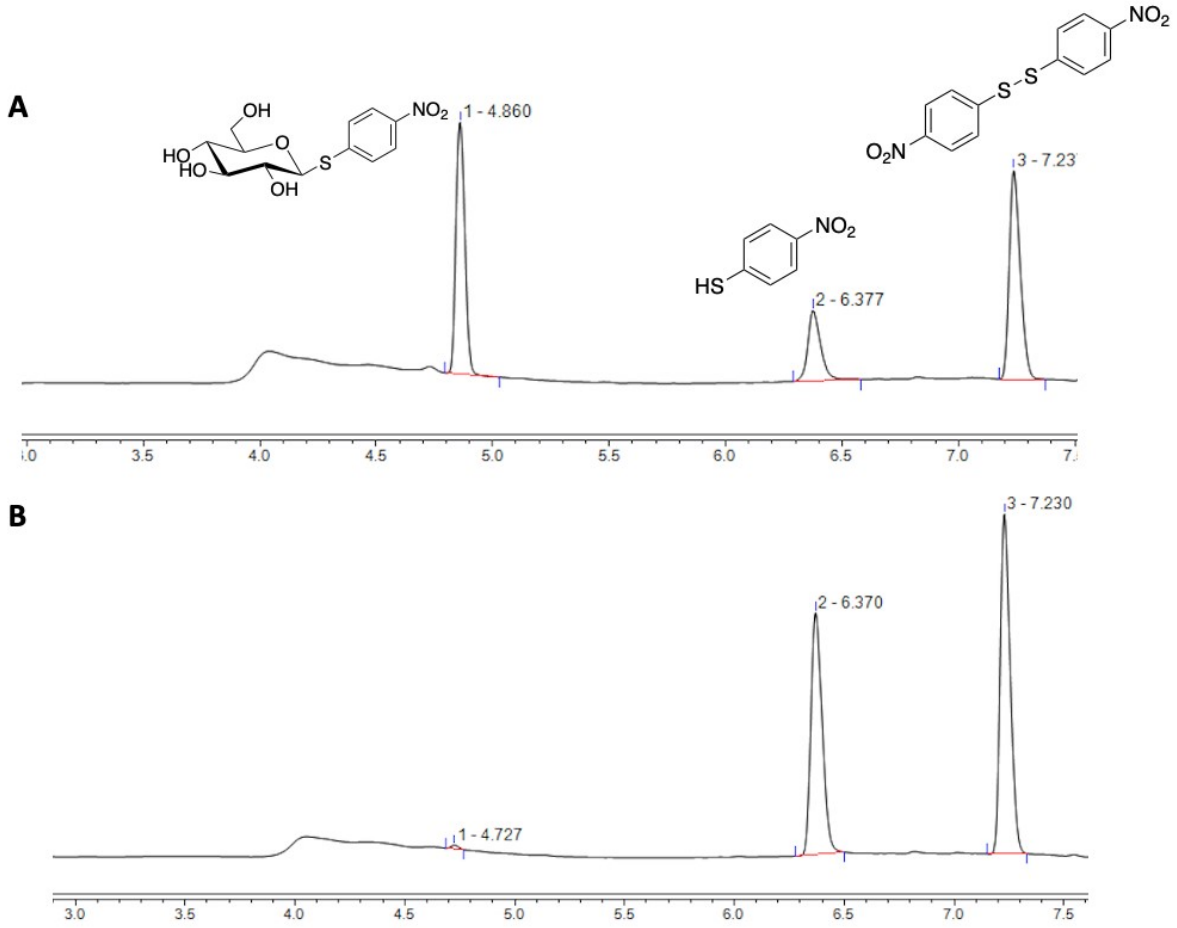


Figure S13: HPLC chromatogram of the reaction of α-D-glucopyranosyl fluoride with *p*-nitrothiophenol (*p*NT) at 25°C for 24h in 50 mM bicarbonate buffer pH 9.4 containing 30 % DMSO, 24h, 1 mg/mL enzyme, 1 mM sugar donor, 20 mM β-mercaptoethanol and 10 mM thiol acceptor, to give *p*-nitrophenyl-β-D-thioglucopyranoside as product **A)** In the presence of the *Hor*GH1 M299R/E166A/E354G mutant and **B)** without enzyme. The first peak at R_t= 4.860 min corresponds to the thioglycosidic product, the second peak at R_t= 6.377 min to the thiol donor and the third peak at R_t=7.23 min to the disulfide version of the thiol donor. No product peak is seen in the absence of the enzyme. In the presence of *Hor*GH1, *Hor*GH1 M299R, *Hor*GH1 E354G and *Hor*GH1 M299R/E354G, chromatograms looked the same as **Figure S13 B**. Chromatograms with other thiol acceptors followed the same pattern of thioglycosidic product, thiol acceptor, and thiol disulfide as **Figure S13 A**, but with different retention times.

1.5.11. Determination of conversion *via* ^{1}H NMR

Conversion of products **3e-f** were determined by ^{1}H-NMR (Bruker Ascend™ 300 (300 MHz) spectrometer) using the integrals of the anomeric protons of the starting materials and product. Reactions were performed as previously described (section 1.5.7. Screening of the thiols accepted by the *Hor*GH1 M299R/E166A/E354G variant) but in deuterated solvents and on a 10 mM scale. Bicarbonate buffer 50 mM pH 9.8 and the enzyme (1 mg/mL) were freeze-dried and redissolved in D_2O, while α-D-glucopyranosyl fluoride was directly dissolved in D_2O. The respective thiol acceptor (*p*-aminothiophenol or 2-mercaptopyridine) and β-mercaptoethanol were dissolved in *d*$_6$-DMSO. ^{1}H NMR spectra were acquired at different time points with 64 scans. Conversion was determined based on the ratio between the integrals of anomeric proton of the product and the sum of the integrals of the anomeric protons of the starting material and product according to the following equation:

$$X\,[\%] = \frac{A_{thioglycoside}}{A_{thioglycoside} + A_{\alpha-D-glucopyranosyl\ fluoride}} \qquad (4)$$

With $A_{thioglycoside}$ representing the integral of the anomeric proton of the thioglycoside product and $A_{\alpha-D-glucopyranosyl\ fluoride}$ the integral of the starting material (**Figure S13 B)**.

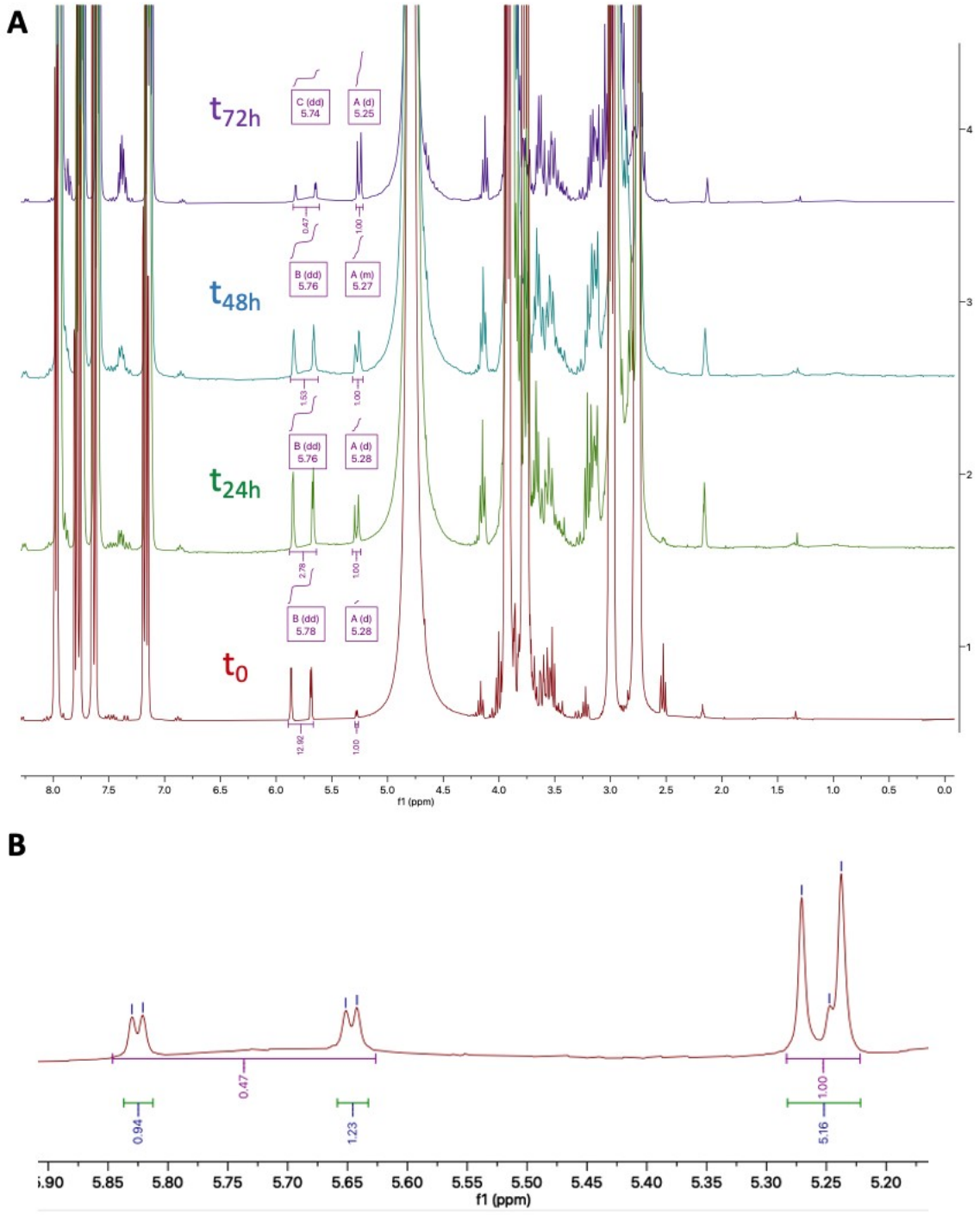
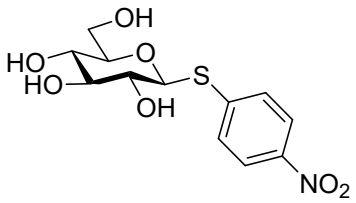


Figure S14: ^{1}H NMR spectra of the reaction of α-D-glucopyranosyl fluoride with 2-mercaptopyridine over time at 25 °C in 50 mM bicarbonate buffer pH 9.4 in D_2O containing 30 % d_6-DMSO, 1 mg/mL enzyme, 10 mM sugar donor, 200 mM β-mercaptoethanol and 100 mM thiol acceptor in a total volume of 1 mL (**A**) and integrals of relevant peaks used to determine product conversion after 72h (**B**) No product peak was observed in the absence of the enzyme. Chemical shifts for the anomeric protons of the starting material and product are provided: δ_H (300 MHz, d_6-DMSO) 5.25 (1H, d, $J_{1,2}$ 9.9 Hz, H-1), 5.65-5.83 (1H, dd, $J_{1\text{-}F}$ 53.7 Hz, $J_{1\text{-}2}$ 2.8 Hz, H-1)

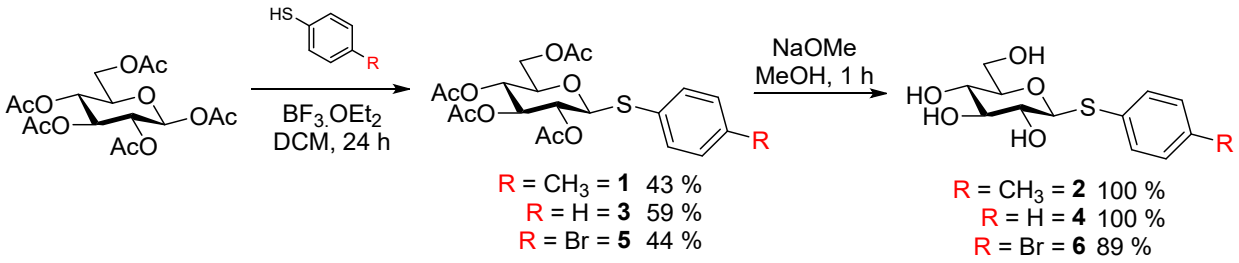
1.6. Synthesis of the thioglycoside standards

Reactions conducted at 0 °C were cooled by means of an ice bath. Solvent was removed under reduced pressure using a Buchi™ rotary evaporator. Reagents were used as supplied without further purification unless otherwise stated. Unless stated, reactions were performed under an atmosphere of nitrogen. Thin Layer Chromatography (t.l.c.) was carried out on Merck Silica Gel 60F254 aluminium-backed plates. Visualisation of the plates was achieved using a UV lamp (λmax = 254 or 365 nm). Flash column chromatography was carried out using Silicycle SiliaFlash® P60 silica (230-400 mesh). Proton and carbon nuclear magnetic resonance (δ_H, δ_C) spectra were recorded on a Bruker Ascend™ 300 (300MHz) spectrometer. All chemical shifts are quoted on the δ-scale in ppm using the residual solvent as an internal standard. ^{1}H and ^{13}C spectra were assigned using COSY, DEPT, HSQC, and HMBC. High resolution mass spectra were recorded a Bruker FT-ICR mass spectrometer using electrospray ionisation (ESI). *M/z* values are reported in Daltons.

1.6.1. *p*-Nitrophenyl ß-D-glucopyranoside 1

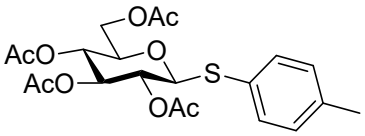


D-Glucose (1.0 g, 5.55 mmol), Et_3N (6.90 mL, 50.0 mmol), and 4-nitrothiophenol (4.30 g 27.8 mmol) were stirred in H_2O/MeCN (1:1, 10 mL). The mixture was cooled to 0 °C. DMC (2.80 g, 16.7 mmol) was then added portionwise. The mixture was allowed to stir for 2 h, at which time, the mixture was diluted with water (40 mL). The aqueous phase was washed with DCM (2 x 50 mL) and then concentrated *in vacuo*. Purification of the residue by flash column chromatography (DCM/MeOH, 7:1) and recrystallisation (MeCN/Et_2O) gave the title compound as a pale yellow solid (145 mg, 8 %); δ_H (300 MHz, D_2O) 3.46-3.52 (2H, m, H-2, H-3), 3.56-3.62 (2H, m, H-4, H-5), 3.76 (1H, dd, $J_{5,6}$ 5.7 Hz, $J_{6,6'}$ 12.5 Hz, H-6), 3.93 (1H, dd, $J_{5,6'}$ 2.2 Hz, H-6'), 5.07 (1H, d, $J_{1,2}$ 9.6 Hz, H-1), 7.67 (2H, d, *J* 9.0 Hz, Ar-H), 8.22 (2H, d, *J* 9.0 Hz, Ar-H); HRMS (ESI) Cald. for $C_{12}H_{15}NO_7SNa^+$ (M + Na^+) 340.0461. Found 340.0458.



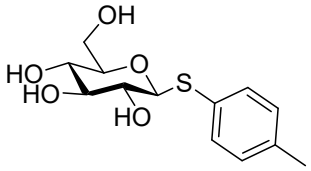
Scheme S1: Synthesis of aryl thioglycosides

1.6.2. Toluoyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside 2



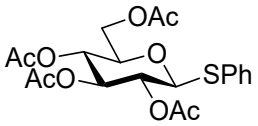
1,2,3,4,6-Penta-*O*-acetyl-ß-D-glucopyranoside (3.0 g, 7.69 mmol) and toluoyl thiol (6.4 g, 51.5 mmol) were stirred in dry DCM (70 mL) and the reaction mixture was cooled to 0 °C. $BF_3.OEt_2$ (6.7 mL, 54.6 mmol) was added dropwise and the reaction mixture was allowed to warm to room temperature. After 24 h, the mixture was diluted with DCM, washed with 1 M aq. NaOH (20 mL), sat. aq. $NaHCO_3$ (50 mL), brine (50 mL), dried (Na_2SO_4), filtered and concentrated *in vacuo*. Recrystallisation (heptane/EtOAc, 2:1) gave toluoyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside **2** as a white crystalline solid (1.5 g, 43 %); δ_H (300 MHz, $CDCl_3$) 1.99, 2.01, 2.08, 2.09 (12H, 4 x s, 4 x CO_2CH_3), 2.35 (3H, s, $ArCH_3$), 3.70 (1H, m, H-5), 4.19 (2H, m, H-6, H-6'), 4.63 (1H, d, $J_{1,2}$ 10 Hz, H-1), 4.93 (1H, at, *J* 10 Hz, H-2), 5.02 (1H, at, *J* 9.7 Hz, H-4), 5.21 (1H, at, *J* 9.3 Hz, H-3), 7.13 (2H, m, Ar-H), 7.39 (2H, m, Ar-H); HRMS (ESI) Cald. For $C_{21}H_{26}O_9SNa^+$ (M + Na^+) 477.1190. Found 477.1202.

1.6.3. Toluoyl ß-D-thioglucopyranoside 3



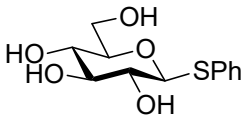
Toluoyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside **2** (1.0 g, 2.20 mmol) was stirred in dry MeOH (10 mL) and NaOMe (50 µL of a 25 wt% solution in MeOH, 0.22 mmol) was added. The reaction was allowed to stir for 2 h, at which time, the mixture was neutralised using Dowex® 50WX8, filtered, and concentrated *in vacuo*. Trituration of the residue with Et_2O and filtration of the resulting solid gave toluoyl ß-D-thioglucopyranoside **3** as a white powder (630 mg, 100 %); δ_H (300 MHz, CD_3OD) 2.31 (3H, s, $ArCH_3$), 3.17 (1H, dd, $J_{2,3}$ 8.3 Hz, $J_{1,2}$ 9.7 Hz, H-2), 3.25-3.39 (3H, m, H-3, H-4, H-5), 3.65 (1H, m, H-6), 3.86 (1H, dd, $J_{6,6'}$ 12.1 Hz, $J_{5,6}$ 1.8 Hz), 4.50 (1H, d, $J_{1,2}$ 9.7 Hz, H-1), 7.12 (2H, m, Ar-H), 7.46 (2H, m, Ar-H); HRMS (ESI) Cald. for $C_{13}H_{17}O_5S^-$ ($M - H^+$) 285.0802. Found 285.0798.

1.6.4. Phenyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside 4



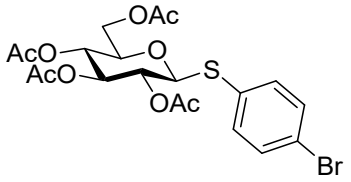
Glucose pentaacetate (1.0 g, 2.56 mmol) and thiophenol (360 µL, 3.59 mmol) were stirred in dry DCM (2 mL) and the mixture was cooled to 0 °C. $BF_3.OEt_2$ (920 µL, 7.43 mmol) was added dropwise and the mixture was allowed to warm to room temperature. After 16 h, the mixture was diluted with DCM (50 mL), washed with NaOH (10 mL of a 1 M aq. solution), $NaHCO_3$ (20 mL of a saturated solution), brine (20 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The product was recrystallised (heptane/EtOAc, 2:1) filtered, and allowed to air-dry to give the phenyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside **4** as a white crystalline solid (760 mg, 59 %); δ_H (300 MHz, $CDCl_3$) 1.99, 2.02, 2.08, 2.09 (12H, 4 x s, 4 x CH_3CO_2), 3.73 (1H, ddd, $J_{4,5}$ 10.1 Hz, $J_{5,6}$ 2.6 Hz, $J_{5,6'}$ 5.0 Hz, H-5), 4.15-4.26 (2H, m, H-6, H-6'), 4.71 (1H, d, $J_{1,2}$ 10.1 Hz, H-1), 4.99 (1H, at, *J* 9.4 Hz, H-2), 5.04 (1H, at, *J* 9.4 Hz, H-4), 5.23 (1H, at, *J* 9.7 Hz, H-3), 7.28-7.34 (3H, m, Ar-H), 7.48-7.51 (2H, m, Ar-H); HRMS (ESI) Cald. for $C_{20}H_{24}O_9SNa$ ($M + Na^+$) 463.1033. Found 463.1022.

1.6.5. Phenyl thio-β-D-glucopyranoside 5



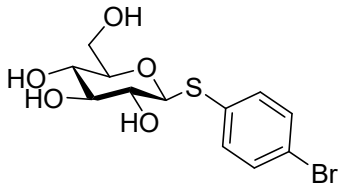
Phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **4** (500 mg, 1.14 mmol) was dissolved in anhydrous MeOH (5 mL) and stirred under N_2 atmosphere. NaOMe (25 µL of a 25 wt% solution in MeOH, 0.114 mmol) was then added. The reaction mixture was allowed to stir at rt until after 1 h, t.l.c (petrol:ethyl acetate, 1:1) indicated complete consumption of starting material (R_f 0.9) and formation of a single product (R_f 0). The reaction mixture was neutralized using Dowex® 50WX8 resin. The resin was filtered and the filtrate was concentrated to afford phenyl 1-thio-β-D-glucopyranoside **5** (310 mg, 100 %) as a white solid, δ_H (300 MHz, CD_3OD) 3.21 (1H, dd, $J_{1,2}$ 9.7 Hz, $J_{2,3}$ 8.5 Hz, H-2), 3.28-3.41 (3H, m, H-3, H-4, H-5), 3.67 (1H, dd, $J_{6,6'}$ 12.2 Hz, $J_{5,6}$ 5.3 Hz, H-6), 3.87 (1H, dd, $J_{6,6'}$ 12.1 Hz, $J_{5,6'}$ 1.8 Hz, H-6'), 4.60 (1H, d, $J_{1,2}$ 9.7 Hz, H-1), 7.22-7.33 (3H, m, Ar-H), 7.54-7.58 (2H, m, Ar-H); HRMS (ESI) Calcd. For $C_{12}H_{16}O_5S^-$ ($M - H^+$) 271.0646. Found 271.0641.

1.6.6. 4-Bromophenyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside 6



Glucose pentaacetate (1.0 g, 2.56 mmol) and 4-bromothiophenol (681 mg, 3.59 mmol) were stirred in dry DCM (2 mL) and the mixture was cooled to 0 °C. $BF_3.OEt_2$ (920 µL, 7.43 mmol) was added dropwise and the mixture was allowed to warm to room temperature. After 16 h, the mixture was diluted with DCM (50 mL), washed with NaOH (10 mL of a 1 M aq. solution), $NaHCO_3$ (20 mL of a saturated solution), brine (20 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The product was recrystallised (heptane/EtOAc, 2:1) and the solid was filtered and allowed to air-dry to give 4-bromophenyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside **6** as a white crystalline solid (580 mg, 44 %); δ_H (300 MHz, $CDCl_3$) 2.02, 2.04, 2.11, 2.11 (12H, 4 x s, 4 x CH_3CO_2), 3.74 (1H, ddd, $J_{4,5}$ 10.0 Hz, $J_{5,6}$ 4.6 Hz, $J_{5,6'}$ 3.0 Hz, H-5), 4.16-4.27 (2H, m, H-6, H-6'), 4.68 (1H, d, $J_{1,2}$ 10.0 Hz, H-1), 4.96 (1H, at, *J* 9.2 Hz, H-2), 5.04 (1H, at, *J* 9.8 Hz, H-4), 5.23 (1H, at, *J* 9.3 Hz, H-3), 7.28-7.39 (4H, m, Ar-H); HRMS (ESI) Cald. for $C_{20}H_{23}BrO_9SNa^+$ ($M + Na^+$) 541.0138. Found 541.0135.

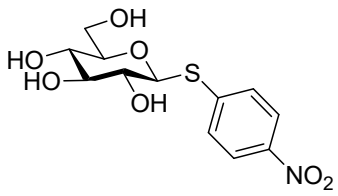
1.6.7. 4-Bromophenyl thio-β-D-glucopyranoside 7

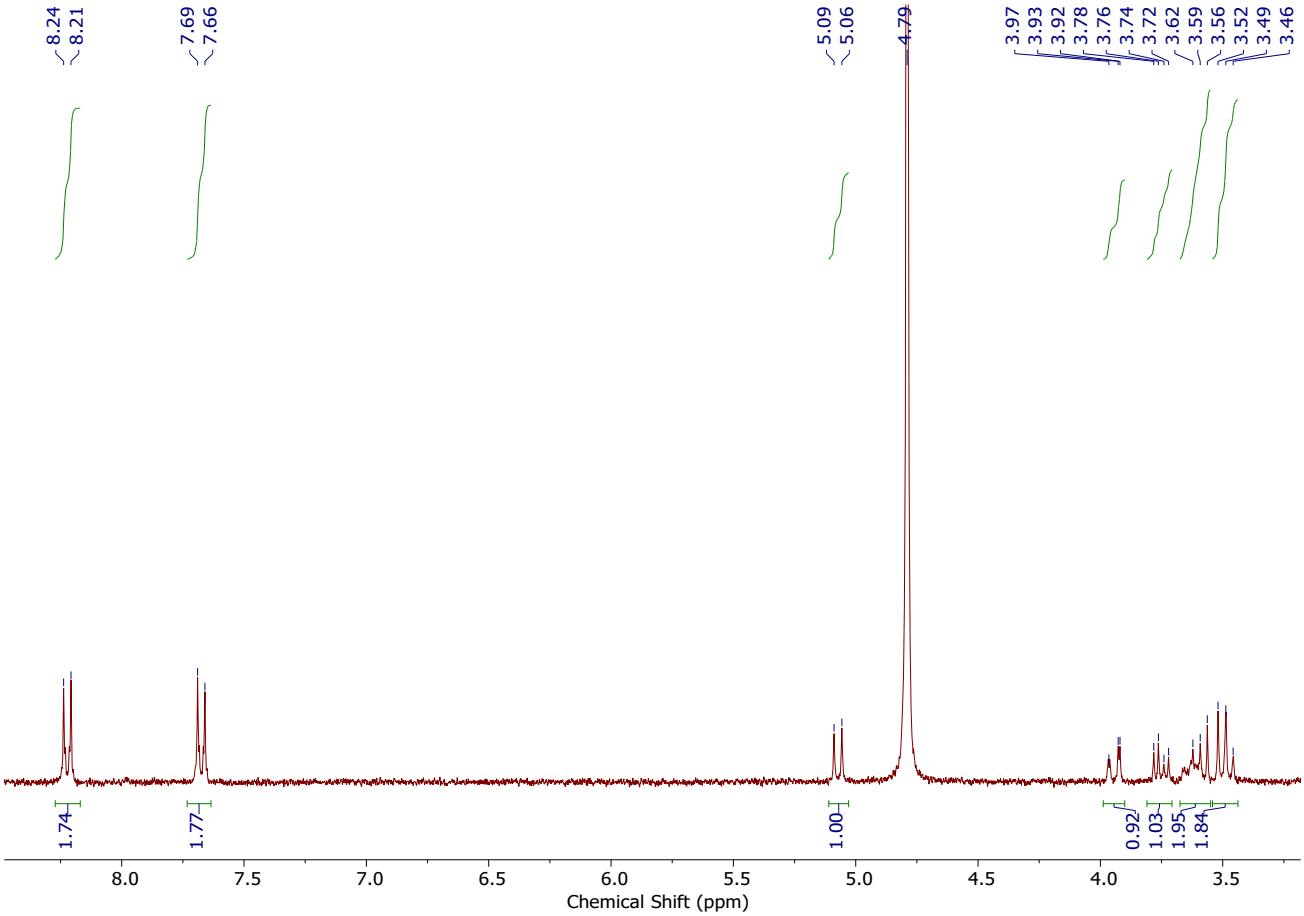


4-Bromophenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **6** (500 mg, 0.96 mmol) was dissolved in anhydrous MeOH (5 mL) and stirred under N_2 atmosphere. NaOMe (21 µL of a 25 wt% solution in MeOH, 0.11 mmol) was then added. The reaction mixture was stirred at rt until after 1 h, t.l.c (petrol:ethyl acetate, 1:1) indicated complete consumption of starting material (R_f 0.9) and formation of a single product (R_f 0). The reaction mixture was neutralized using Dowex® 50WX8 resin. The resin was filtered and the filtrate was concentrated to afford 4-bromophenyl 1-thio-β-D-glucopyranoside **7** (300 mg, 89 %) as a white solid; δ_H (300 MHz, $CDCl_3$) 3.16-3.38 (4H, m, H-2, H-3, H-4, H-5), 3.66 (1H, dd, $J_{6,6'}$ 12.1 Hz, $J_{5,6}$ 5.3 Hz, H-6), 3.87 (1H, dd, $J_{6,6'}$ 12.1 Hz, $J_{5,6'}$ 2.0 Hz, H-6'), 4.58 (1H, d, $J_{1,2}$ 9.7 Hz, H-1), 7.43-7.50 (4H, m, Ar-H); HRMS (ESI) Calcd. For $C_{12}H_{14}O_5S^-$ ($M - H^+$) 348.9751. Found 348.9745.

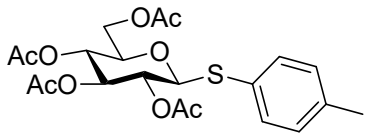
2. Spectral Data

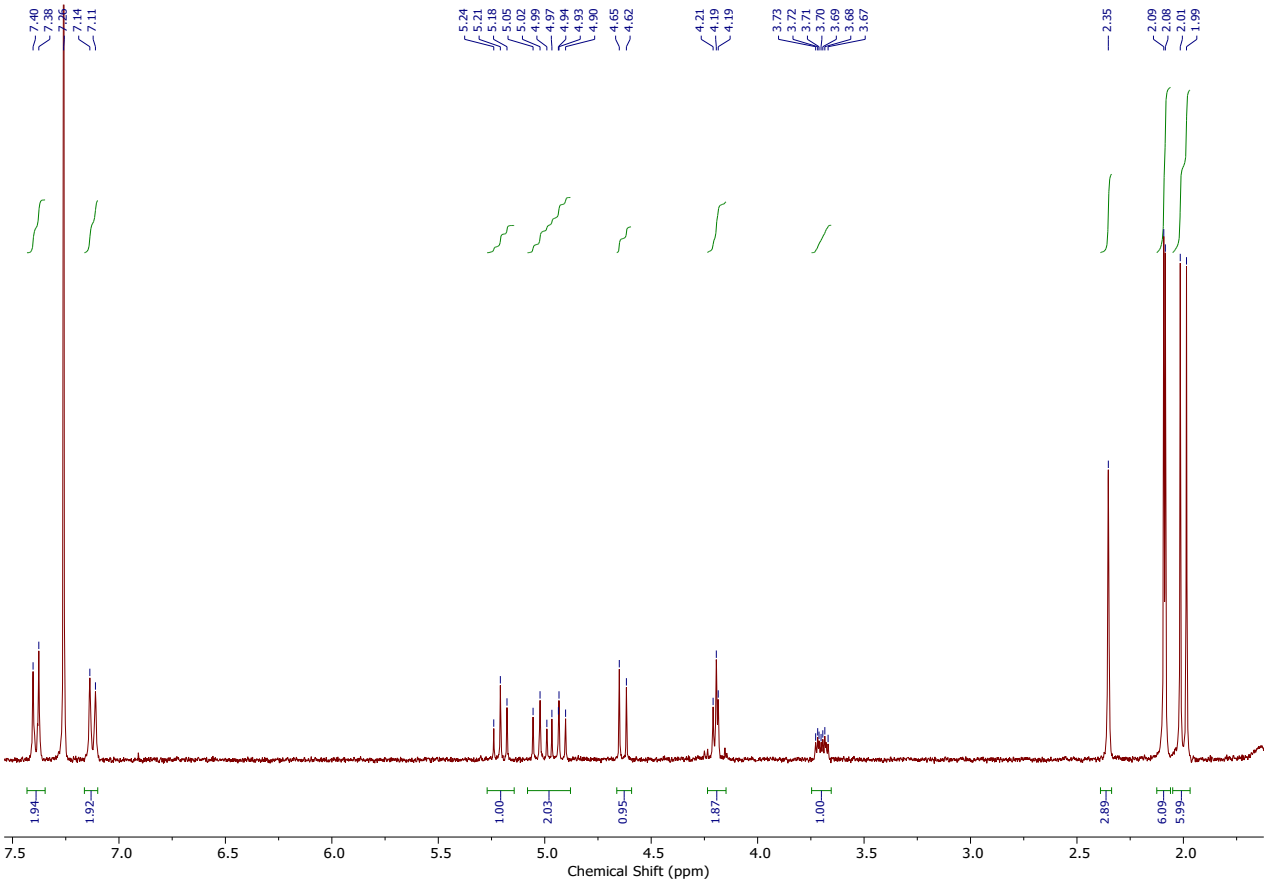
2.1. *p*-Nitrophenyl ß-D-glucopyranoside 1



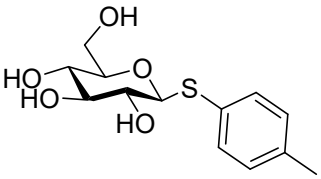


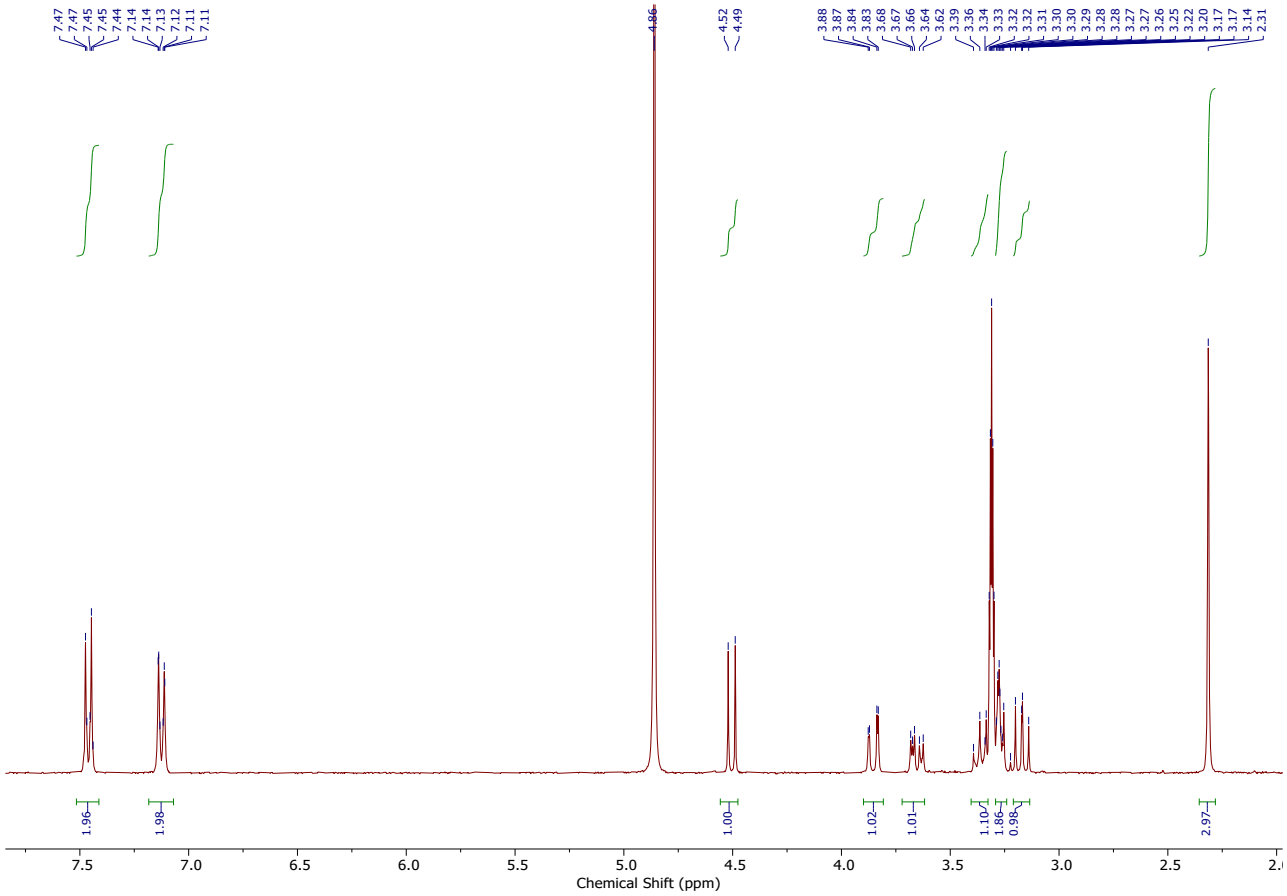
2.2. Toluoyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside 2



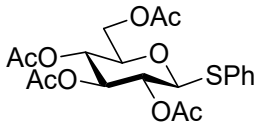


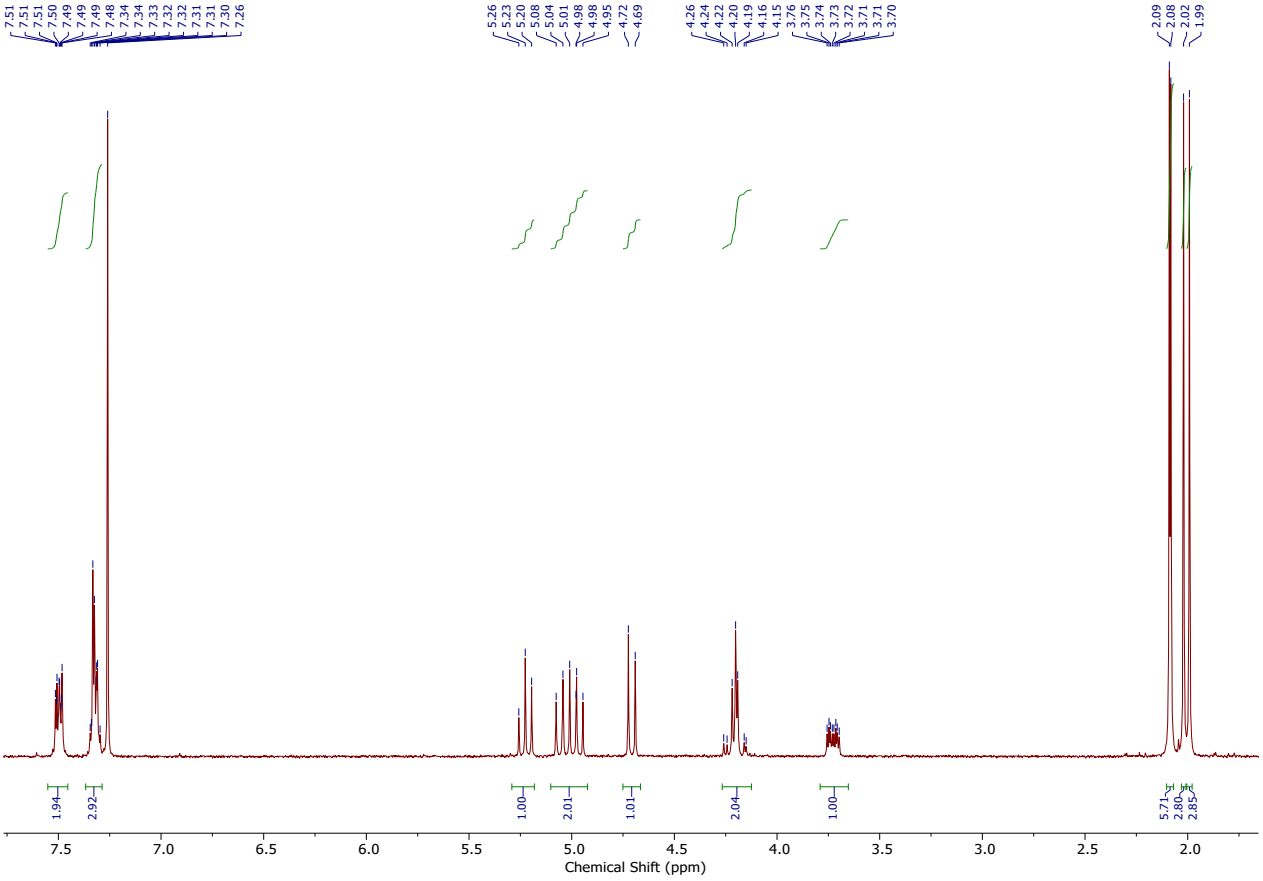
2.3. Toluoyl ß-D-thioglucopyranoside 3



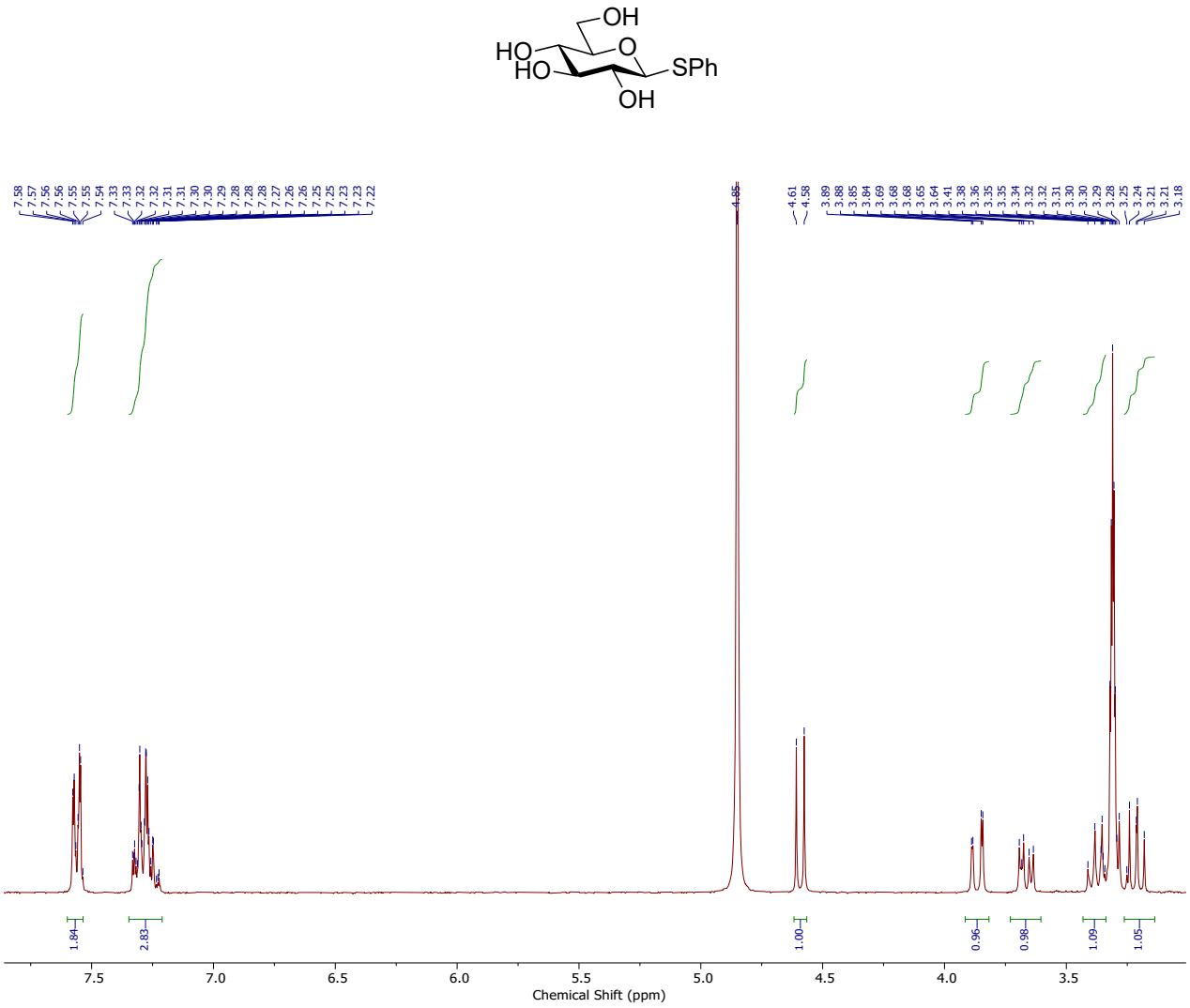


2.4. Phenyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside 4

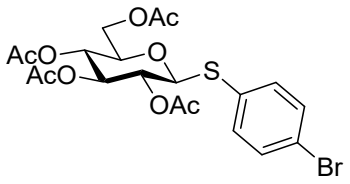


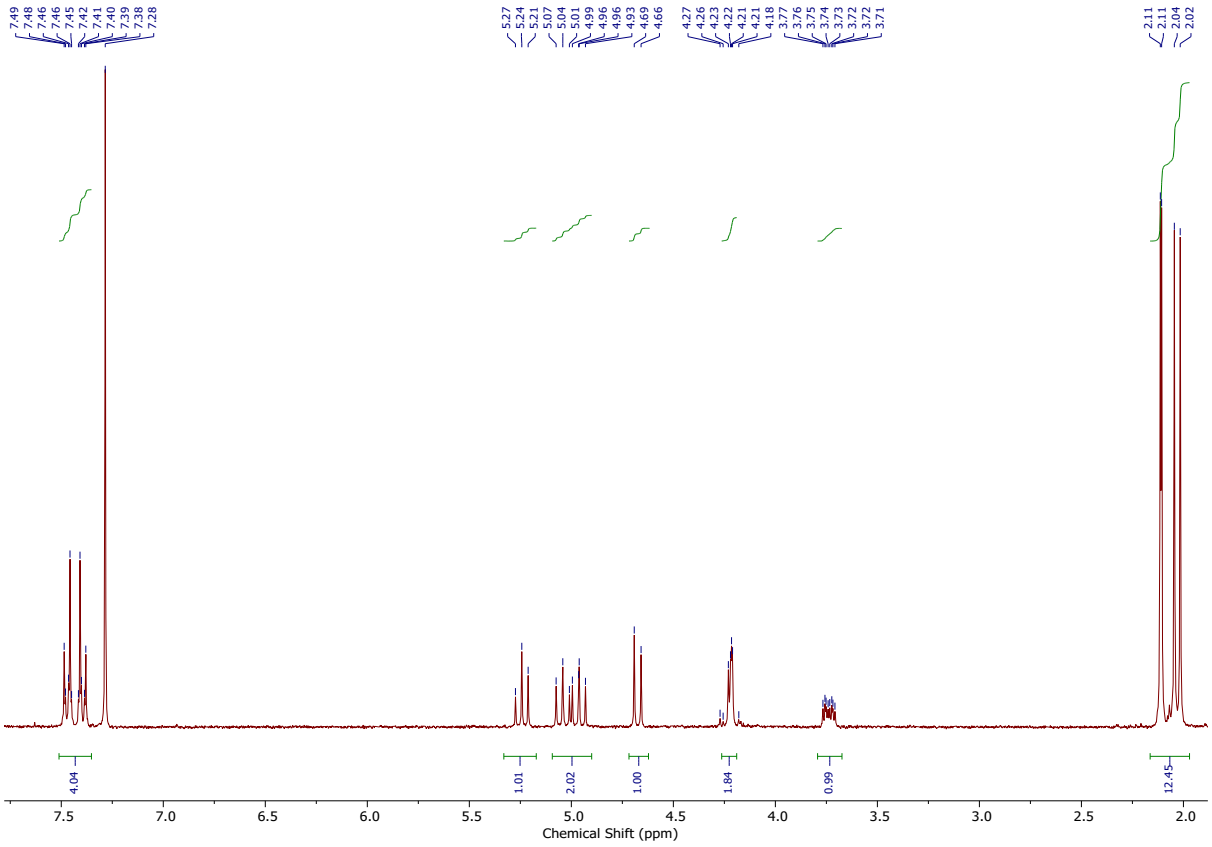


2.5. Phenyl thio-β-D-glucopyranoside 5

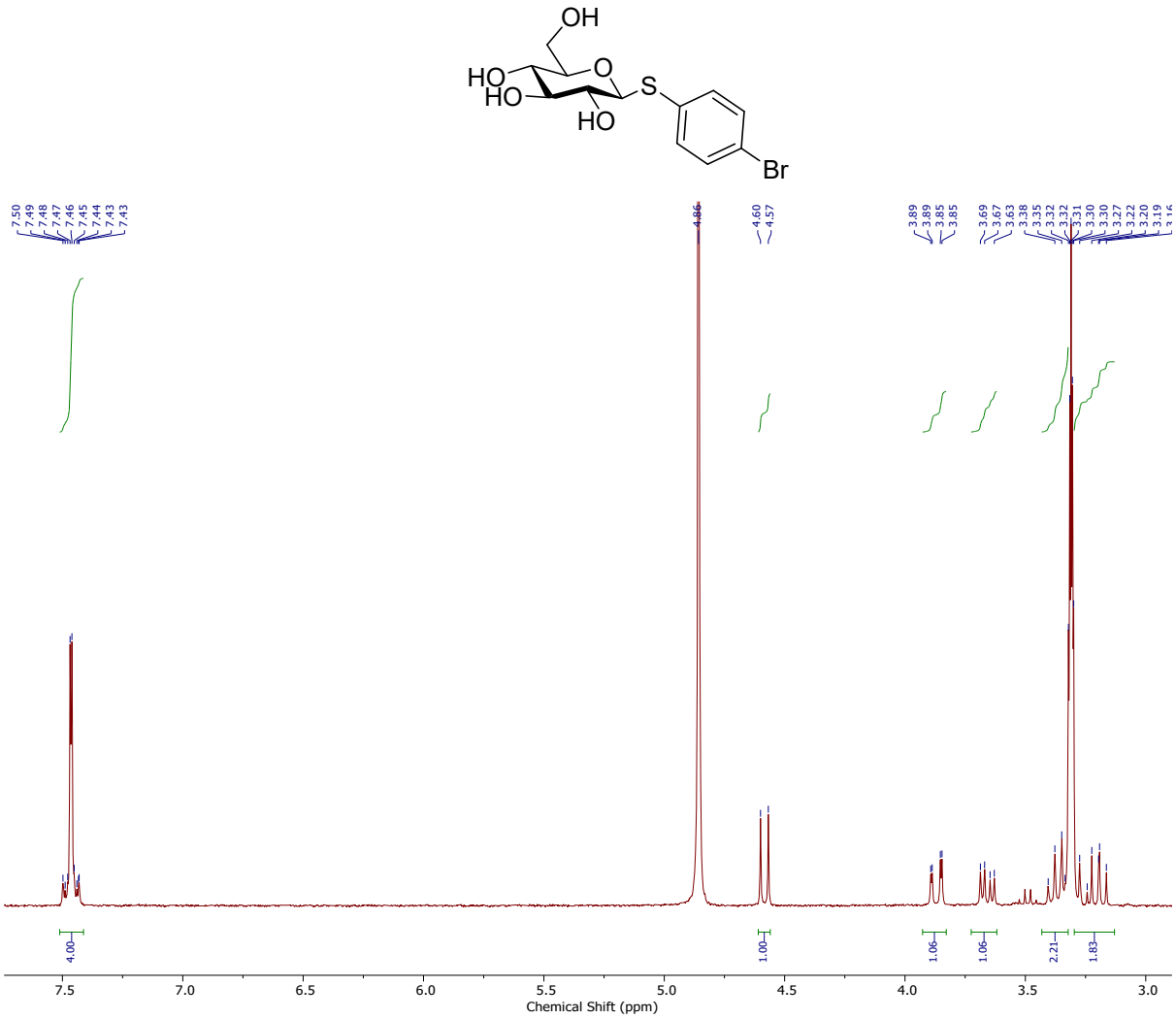


2.6. 4-Bromophenyl 2,3,4,6-tetra-*O*-acetyl-ß-D-thioglucopyranoside 6





2.7. 4-Bromophenyl thio-β-D-glucopyranoside 7



3. Supplementary references

1. N. Almulhim, N. R. Moody, F. Paradisi. *Appl. Microbiol. Biotechnol*., 2020, **104**(10), 4407 4415.

2. M.R. Wilkins, E. Gasteiger, A. Bairoch, J.C. Sanchez, K.L. Williams, R.D. Appel, D.F. Hochstrasser, *Methods Mol Biol*., 1999, **112,** 531-552.

3. Very simple K_M V_{max} toolkit. http://www.ic50.tk/kmvmax.html (Accessed 27.09.2022).

4. T. Tanaka, T. Matsumoto, M. Noguchi, A. Kobayashi, S. Shoda, *Chem. Lett.*, 2009, **38**(5), 458-459

5. N. Yoshida, M. Noguchi, T. Tanaka, T. Matsumoto, N. Aida, M. Ishihara, A. Kobayashi, S.I. Shoda. *Chem Asian J*., 2011, **6**(7), 1876-1885.