

## Electronic Supplementary Information

### Asymmetric trehalose analogues to probe disaccharide processing pathways in mycobacteria

Hadyn L. Parker<sup>a</sup>, Ruben M. F. Tomás<sup>b</sup>, Christopher M. Furze<sup>a</sup>, Collette S. Guy<sup>a,b</sup>, Elizabeth Fullam<sup>\*a</sup>

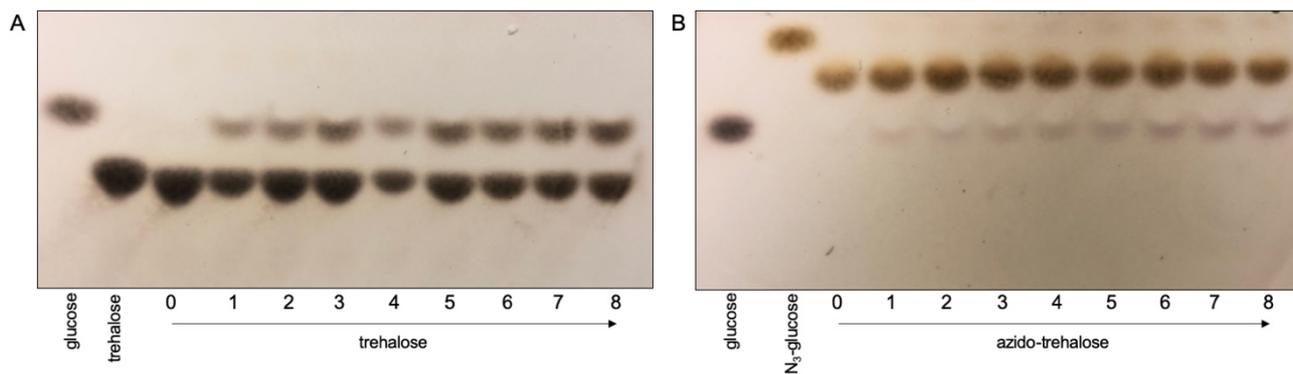
<sup>a</sup>School of Life Sciences, University of Warwick, Coventry, CV4 7AL, United Kingdom

<sup>b</sup>Department of Chemistry, University of Warwick, Coventry, CV4 7AL, United Kingdom

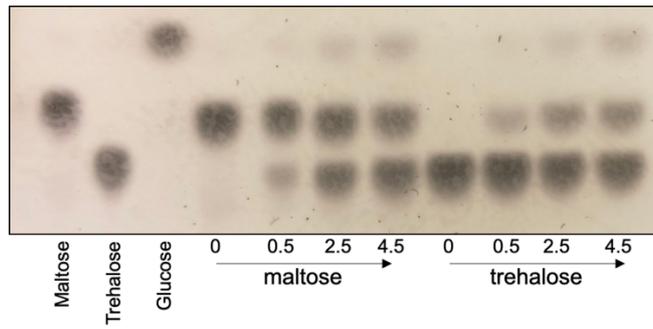
\*To whom correspondence should be addressed: Elizabeth Fullam, School of Life Sciences, University of Warwick, Coventry, CV4 7AL, United Kingdom;  
[e.fullam@warwick.ac.uk](mailto:e.fullam@warwick.ac.uk); Tel. +44 (0)2476 574239

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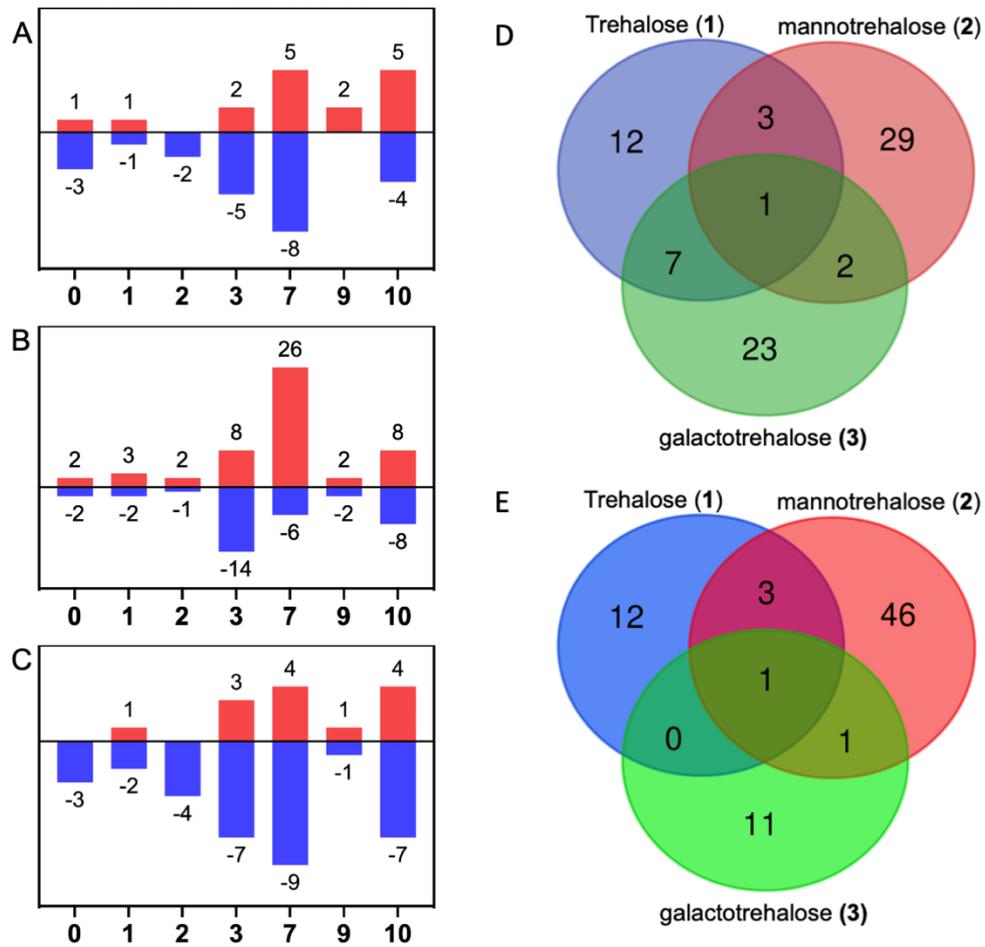
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**Fig. S1.** Activity of the *M. smegmatis* trehalase enzyme with trehalose (1) and azido-trehalose (4) over an 8 h time period. The numbers below the lane indicate the time-point in h.



**Fig. S2.** Activity of the *M. smegmatis* TreS enzyme with maltose and trehalose (1) over a 4.5 h time period. The numbers below the lane indicate the time-point in h.



**Fig. S3.** Whole cell proteomic analysis of *M. bovis* BCG in response to **1-3**. A-C) Proteins that are up (red) or down (blue) regulated in response to trehalose (**1**) (A), mannotrehalose (**2**) (B) or galactotrehalose (**3**) (C). Functional category assigned by Mycobrowser: 0: virulence, detoxification & adaption, 1: lipid metabolism, 2: information pathways, 3: cell wall & cell wall processes, 7: intermediary metabolism and respiration, 9: regulatory proteins, 10: conserved hypotheticals. D-E) Venn diagrams showing the number of proteins that are (D) up-regulated or (E) down-regulated in response to exposure of **1-3**.

## Experimental

### General Experimental Section

Materials and reagents were purchased from Sigma-Aldrich, unless specified, and were used without further purification. Carbohydrates were obtained from Carbosynth unless otherwise stated, 6-azido-6-deoxy-D-mannose was obtained from CarboSynUSA.

### Expression and purification of TreT

The trehalose synthase (TreT) enzyme from *Thermoproteus tenax* was overexpressed and purified as described previously.<sup>1</sup> In brief, *E. coli* Top 10 were transformed with the *treT\_pBADHisA* expression plasmid (a gift from Dr B Swarts (Central Michigan University, USA)) and grown at 37 °C to an optical density at 600 nm (OD<sub>600</sub>) of 0.6 in Terrific Broth (Difco) supplemented with 100 µg/mL ampicillin. Protein production was induced with 1 mM L-arabinose and the cultures were grown at 37 °C overnight with shaking (180 rpm). The cells were harvested (4,000 *x g*, 30 min, 4 °C) and the pellets were resuspended in PBS and frozen at -80 °C until further use. The frozen pellets were resuspended in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 20 mM imidazole, pH 8.0, (buffer A), Complete Protease Inhibitor Cocktail (Pierce) and lysozyme (60 mg) were added and the cells sonicated on ice (Sonicator Ultrasonic Liquid Processor XL; Misonix). Following sonication, the cells were centrifuged (39,000 *x g*, 30 min, 4 °C) and the supernatant was filtered (0.45 µm filter, EMD Millipore) and loaded onto a pre-equilibrated HisPur Ni<sup>2+</sup>-affinity resin (Thermo Scientific). The column was washed with buffer A (5 column volumes) and the recombinant TreT protein was eluted from the Ni<sup>2+</sup> resin with increasing concentrations of imidazole. Fractions containing the TreT protein, as determined by SDS-PAGE, were dialysed at 4 °C for 12 h against 50 mM HEPES, 300 mM NaCl, pH 8.0 (buffer B) and purified further using size exclusion chromatography (Superdex 200 16/60 column (GE Healthcare)). Fractions containing TreT were pooled, and the protein concentrated to 1.5-5 mg/mL (Amicon, 10 kDa MWCO) and stored at 4 °C. The identity of the protein was confirmed by tryptic digest and nanoLC-ESI-MS/MS (WPH Proteomics Facility, University of Warwick).

### Chemoenzymatic synthesis of trehalose derivatives

The enzymatic reaction contained either D-mannose (45 mM), D-galactose (30 mM), 6-azido-6-deoxy-D-glucose (30 mM), 6-azido-6-deoxy-D-mannose (30 mM) or 6-azido-6-deoxy-D-galactose (30 mM), UDP-Glucose (either 30, 20, 20, 20 or 20 mM respectively in relation to each carbohydrate), MgCl<sub>2</sub> (40 mM), and TreT (300 µg/mL) in 50 mM HEPES, 300 mM NaCl, pH 8.0, and was incubated at 70 °C for 2 h, with shaking (300 rpm). Reactions were quenched by the addition of an equal volume of cold acetone, cooled at -20 °C for 2 hours and centrifuged (18,000 *x g*, 4 °C, 20 min). The supernatant was collected and concentrated in vacuum and the resulting residue was resuspended in water and purified by size exclusion using Biogel-P2 column (Bio-Rad) using water as an eluent. The fractions were monitored by TLC (5:3:2 *n*-butanol/ethanol/water), stained with 5% H<sub>2</sub>SO<sub>4</sub> in ethanol and heated to visualise spots containing sugars. The fractions containing the desired product were concentrated *in vacuo* and purified further by HPLC where necessary (amino-column: 5

$\mu\text{m}$ , 250 x 4.6 mm (Phenomenex Luna) at 40 °C; mobile phase: 80% acetonitrile in water, HPLC grade; flow rate: 2.5 mL/min; detection: refractive index).

### **$^1\text{H}$ NMR, $^{13}\text{C}$ NMR and MS data**

Proton ( $^1\text{H}$ ) and carbon ( $^{13}\text{C}$ ) NMR spectra were obtained at 298 K.  $^1\text{H}$  NMR were recorded on Bruker DPX-400 and DPX-500 instruments as indicated.  $^{13}\text{C}$  DEPT NMR were collected on a Bruker DPX-500 instrument. NMRs were fully assigned using COSY, HSQC and HMBC.  $^1\text{H}$  NMR chemical shifts are quoted in parts per million (ppm), using the residual solvent as the internal standard ( $^1\text{H}$  D<sub>2</sub>O = 4.79 ppm). Coupling constants ( $J$ ) are reported in hertz (Hz) with the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; br, broad. Low resolution mass spectra were recorded on a Bruker Esquire 2000 spectrometer using electrospray ionisation (ESI) and high resolution mass spectra were recorded on a Bruker HCT Ultra spectrometer.  $m/z$  values are reported in Daltons (Da).

#### **$\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside: Mannotrehalose (2)**

Product was obtained as a white solid (6.1 mg, 59.5%).  $^1\text{H}$  NMR (400 MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  5.10 (1H, d,  $J = 3.5$  Hz, C<sup>1</sup>H), 5.04 (1H, d,  $J = 1.5$  Hz, C<sup>1</sup>H), 3.91 (1H, dd,  $J = 3.5, 2.0$  Hz), 3.86 (1H, dd,  $J = 9.5, 3.5$  Hz), 3.74 – 3.83 (2H, m), 3.63 – 3.74 (4H, m), 3.52 – 3.63 (3H, m), 3.35 (1H, t,  $J = 9.5$  Hz).  $^{13}\text{C}$  NMR (100 MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  95.0, 93.5 (C<sup>1</sup> and C<sup>1</sup>'), 73.2, 72.6, 72.4, 70.9, 70.2, 70.0, 69.6, 66.7 (C<sup>2</sup>-C<sup>5</sup>), 60.9, 60.5 (C<sup>6</sup>H<sub>2</sub>OH and C<sup>6</sup>H<sub>2</sub>OH).  $m/z$  (ES<sup>-</sup>): [M-H]<sup>-</sup> calcd. for C<sub>12</sub>H<sub>21</sub>O<sub>11</sub><sup>-</sup>, 341.3; found 341.1

#### **$\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside: Galactotrehalose (3)**

Product was obtained as a white solid (3.8 mg, 27.8%).  $^1\text{H}$  NMR (500 MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  5.12 (2H, dd,  $J = 7.5, 4.0$  Hz, C<sup>1</sup>H + C<sup>1</sup>H), 3.98 (1H, dd,  $J = 7.0, 5.5$  Hz), 3.89 – 3.95 (2H, m), 3.82 (1H, dd,  $J = 10.0, 4.0$  Hz), 3.60 – 3.79 (6H, m), 3.56 (1H, dd,  $J = 10.0, 4.0$  Hz), 3.36 (1H, t,  $J = 9.5$  Hz).  $^{13}\text{C}$  NMR (126 MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  93.3, 93.1 (C<sup>1</sup> and C<sup>1</sup>'), 72.5, 72.1, 71.3, 71.0, 69.6, 69.2, 68.9, 67.9 (C<sup>2</sup>-C<sup>5</sup>), 61.2, 60.5 (C<sup>6</sup>H<sub>2</sub>OH and C<sup>6</sup>H<sub>2</sub>OH).  $m/z$  (ES<sup>-</sup>): [M-H]<sup>-</sup> calcd. for C<sub>12</sub>H<sub>21</sub>O<sub>11</sub><sup>-</sup>, 341.3; found 341.1

#### **6-azido-6-deoxy- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside: 6-azido-trehalose (4)**

Product was obtained as a white solid (2.5 mg, 34.2%).  $^1\text{H}$  NMR (500MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  5.10 (2H, app t,  $J = 4.5$  Hz, C<sup>1</sup>H + C<sup>1</sup>H), 3.87 (1H, ddd,  $J = 9.0, 6.0, 2.5$  Hz), 3.70 – 3.80 (4H, m), 3.67 (1H, dd,  $J = 12.0, 5.0$  Hz), 3.53 – 3.62 (3H, m), 3.47 (1H, dd,  $J = 13.5, 6.0$  Hz), 3.36 (2H, td,  $J = 9.5, 3.5$  Hz).  $^{13}\text{C}$  NMR (126MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  93.6, 93.4 (C<sup>1</sup> and C<sup>1</sup>'), 72.5, 72.3, 72.2, 71.0, 70.9, 70.9, 70.4, 69.6 (C<sup>2</sup>-C<sup>5</sup>), 60.5 (C<sup>6</sup>H<sub>2</sub>OH), 50.8 (C<sup>6</sup>H<sub>2</sub>N<sub>3</sub>).  $m/z$  (ES<sup>-</sup>): [M-H]<sup>-</sup> calcd. for C<sub>12</sub>H<sub>20</sub>N<sub>3</sub>O<sub>10</sub><sup>-</sup>, 366.3; found 366.2

#### **6-azido-6-deoxy- $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside: 6-azido-mannotrehalose (5)**

Product was obtained as a white solid (3.3 mg, 22.5%).  $^1\text{H}$  NMR (500 MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  5.10 (1H, d,  $J = 4.0$  Hz, C<sup>1</sup>H), 5.04 (1H, br s, C<sup>1</sup>H), 3.95 – 3.92 (1H, m), 3.87 – 3.80 (2H, m), 3.80 – 3.73 (1H, m), 3.70 – 3.52 (6H, m), 3.48 (1H, dd,  $J = 13.5, 6.5$  Hz), 3.35 (1H, t,  $J = 9.5$  Hz).  $^{13}\text{C}$  NMR (126 MHz, D<sub>2</sub>O)  $\delta_{\text{ppm}}$  95.2, 93.8 (C<sup>1</sup> and C<sup>1</sup>'), 72.6, 72.5, 72.0, 70.8, 70.0, 69.9, 69.5, 67.4 (C<sup>2</sup>-C<sup>5</sup>), 60.5 (C<sup>6</sup>H<sub>2</sub>OH), 51.0 (C<sup>6</sup>H<sub>2</sub>N<sub>3</sub>).  $m/z$  (ES<sup>+</sup>): [M+Na]<sup>+</sup> calcd. for C<sub>12</sub>H<sub>21</sub>N<sub>3</sub>O<sub>10</sub>Na<sup>+</sup>, 390.1119; found 390.1113.

### **6-azido-6-deoxy- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside: 6-azido-galactotrehalose (6)**

Product was obtained as a white solid (2.2 mg, 20.0%).  $^1\text{H}$  NMR (500MHz,  $\text{D}_2\text{O}$ )  $\delta_{\text{ppm}}$  5.13 (1H, d,  $J = 4.0$  Hz,  $\text{C}^1\text{H}$ ), 5.11 (1H, d,  $J = 4.0$  Hz,  $\text{C}^1\text{H}$ ), 4.12 (1H, dd,  $J = 9.0, 4.0$  Hz), 3.88 – 3.94 (2H, m), 3.81 (1H, dd,  $J = 10.0, 4.0$  Hz), 3.75 (3H, m), 3.67 (1H, dd,  $J = 12.0, 5.5$  Hz), 3.56 (1H, dd,  $J = 10.5, 4.0$  Hz), 3.44 – 3.52 (1H, m), 3.32 – 3.39 (2H, m).  $^{13}\text{C}$  NMR (126MHz,  $\text{D}_2\text{O}$ )  $\delta_{\text{ppm}}$  94.1, 93.6 ( $\text{C}^1$  and  $\text{C}^1$ ), 72.5, 72.2, 71.0, 70.0, 69.6, 69.6, 68.8, 67.1 ( $\text{C}^2\text{-C}^5$ ), 60.5 ( $\text{C}^6\text{H}_2\text{OH}$ ), 51.0 ( $\text{C}^6\text{H}_2\text{N}_3$ ).  $m/z$  ( $\text{ES}^+$ ):  $[\text{M}+\text{Na}]^+$  calcd. for  $\text{C}_{12}\text{H}_{21}\text{N}_3\text{O}_{10}\text{Na}^+$ , 390.1119; found 390.1115.

### **Enzymatic reaction of analogues with trehalase from *Mycobacterium smegmatis***

Trehalose or trehalose analogues (30 mM, final concentration) were incubated with trehalase 15A from *M. smegmatis* (NZYTech) (100  $\mu\text{g}/\text{mL}$ ) in 100 mM HEPES, 100 mM  $\text{Na}_2\text{HPO}_4$ , 6 mM  $\text{MgCl}_2$ , pH 7.1 at 37  $^\circ\text{C}$  for 19 hours with shaking (350 rpm). In parallel, control reactions without the *M. smegmatis* trehalase enzyme were assayed. The enzymatic reaction was stopped by heating the reaction at 80  $^\circ\text{C}$  for 10 min followed by centrifuging at 18,000  $\times g$ , for 10 min at 4  $^\circ\text{C}$ . The supernatants were removed and analysed by thin-layer chromatography (TLC) using 5:3:2 *n*-butanol/ethanol/water. Trehalose, azido-trehalose, glucose and azido-glucose were included as standards. The TLC plates were stained with 5%  $\text{H}_2\text{SO}_4$  in ethanol and heated to visualise the sugars.

### **Enzymatic reaction of analogues with trehalose synthase (TreS) from *Mycobacterium smegmatis***

Trehalose or trehalose analogues (20 mM, final concentration) were incubated with TreS (150  $\mu\text{g}/\text{mL}$ ) from *M. smegmatis* (NZYtech) in PBS, 6 mM  $\text{MgCl}_2$ , pH 7.4 at 40  $^\circ\text{C}$  with shaking (350 rpm). Aliquots of the reaction (10  $\mu\text{L}$ ) were taken from 0 – 4.5 h. Ice-cold acetone (20  $\mu\text{L}$ : 2x reaction volume) was added to quench the reaction, incubated at -20  $^\circ\text{C}$  overnight and centrifuged at 18,000  $\times g$ , for 10 min at 4  $^\circ\text{C}$  to remove the TreS enzyme. The supernatants were removed and analysed by thin-layer chromatography (TLC) using 7:3:1 *n*-butanol/pyridine/water. Maltose, trehalose, glucose and azido-trehalose were included as standards. The TLC plates were stained with 5%  $\text{H}_2\text{SO}_4$  in ethanol and heated to visualise the sugars.

### **Bacterial strains and culture conditions**

*Mycobacterium smegmatis* MC<sup>2</sup>155 (ATCC-700084) and *Mycobacterium bovis* BCG (ATCC-35734) were routinely grown at 37  $^\circ\text{C}$  in Middlebrook 7H9 broth (BD Difco) supplemented with 0.2 % glycerol, 0.05 % Tween 80 and 10% albumin-dextrose-catalase (ADC). *M. bovis* BCG was cultured at containment level 2. The gene deletion mutants and complemented strains: *M. smegmatis*  $\Delta\text{lpqY}$ , *M. smegmatis*  $\Delta\text{sugC}$ , *M. smegmatis*  $\Delta\text{sugC}:\text{sugC}_{pMV361}$ , were provided by Professor Rainer Kalscheuer and cultured with the addition of the appropriate antibiotic/s.<sup>2</sup>

## Growth curves

*M. smegmatis* and *M. bovis* BCG were grown to mid-log phase in 7H9/ADC/Tween media in 96-well plates at 37 °C with shaking (180 rpm). To starve the cells, the culture was pelleted by centrifugation (2,900  $\times$  g, 10 min, 4 °C), washed three times in PBS containing 0.05% tyloxapol, resuspended in PBS containing 0.05% tyloxapol and either grown overnight (*M. smegmatis*) or for 24 h (*M. bovis* BCG) at 37 °C, with shaking (180 rpm). Following starvation the cells were centrifuged (2,900  $\times$  g, 10 min, 4 °C), washed three times in PBS containing 0.05% tyloxapol and resuspended in M9 minimal media containing 0.05% tyloxapol to an OD<sub>600</sub> of 0.2, trehalose or trehalose analogues were added (2.9 mM final concentration) and the growth of *M. smegmatis* and *M. bovis* BCG were carried out in 96-well microtitre plates and the optical density at 595 nm (OD<sub>600</sub>) was monitored (Tecan Infinite M200) at regular intervals. For *M. smegmatis* the curves were fitted to the data points using the Lowess fit in GraphPad Prism V8. All experiments were carried out in triplicate.

## Labelling of mycobacteria

*M. smegmatis* was grown to an OD<sub>600</sub> of 0.25, then transferred to a V-bottom 96-well plate (200  $\mu$ L) and either azido-trehalose (**4**) or the azido-trehalose analogues (**5-6**) (100  $\mu$ M) or no sugar control added, incubated at 37 °C with shaking (450 rpm) for 4 hours, then centrifuged (2,600  $\times$  g, 5 min, 4 °C) and washed three times with PBS containing 0.05% Tween 80 (PBST) (3  $\times$  200  $\mu$ L). The cells were resuspended in 7H9/ADC/Tween 80 followed by the addition of the dibenzocyclooctyne-PEG4-tetramethylrhodamine (DBCO-PEG4-TAMRA) dye (26.6  $\mu$ M) and incubated at 37 °C for 2 h in the dark. The cells were then centrifuged (2,600  $\times$  g, 5 min, 4 °C), washed three times with PBST, fixed in 4% paraformaldehyde in PBS and incubated at 37 °C in the dark overnight. The cells were then washed with PBST three times before analysis by fluorescence microscopy or flow cytometry.

## Confocal microscopy

The labelled mycobacteria were spotted onto glass slides that had been prepared with 7H10 media (Middlebrook) containing 1.5% agarose and allowed to air-dry. Cover slips were placed over the sample which was fixed with an adhesive. Microscopy was performed on an LSM 880 confocal microscope (Carl ZEISS) equipped with a Plan-Apochromat 63x/1.40 and 100x/1.40 oil immersion objective lens and equipped with an Airyscan detector. DBCO-PEG4-TAMRA was excited with a 514 nm argon laser (5 %) and detected in the 524-554 nm range. Transmitted light was detected by the T-PMT detector. Fluorescence was detected by either the PMT or Airyscan detectors. Images processed on ZEN 2.3 SP1 FP1, version 14.0.9.201.

## Flow Cytometry

Flow cytometry was performed on a BD Influx™ cell sorter (BD Biosciences) running BD FACS Software™ software and equipped with 355-, 488-, 561-, and 642-nm lasers, detecting up to 24 parameters (21 fluorescence channels, two forward scatter channels and one side scatter). Sample analysis required the use of the 488 nm excitation laser and 580/30 nm filter for DBCO-PEG4-TAMRA detection with 100,000 events recorded. A 100  $\mu$ m nozzle was fitted, operating at a pressure of 20 psi (sheath) and 21.5 psi (sample). Stream

and laser alignment was performed using BD Sphero™ Rainbow Calibration Particles (8 Peaks 3.0-3.4  $\mu\text{m}$ ). Voltage settings applied ensured that untreated control cells appeared at low emission intensities and all treated cells were within the detection range. FlowJo X 10.0.7r2 (Tree Star, Ashland, USA) was used for all statistical analysis and plotting of flow cytometry data. All flow cytometry experiments were performed in triplicate.

## **Proteomics**

### **Preparation of samples for proteomics**

*M. bovis* BCG (10 mL) was grown to an  $\text{OD}_{600}$  of 0.2 in 7H9/Tween/ADC and after which either trehalose (**1**), trehalose analogues **2-3** (1.5 mM, 0.5 mg/mL) or no compound was added. The *M. bovis* BCG cultures were incubated at 37 °C for 48 hours, with shaking (180 rpm). The cells were harvested ( $2,916 \times g$ , 10 min, 4 °C), washed (3 x PBST) and the pellet resuspended in lysis buffer (PBS, 1 mM DTT, 1 mg/mL lysozyme, protease inhibitor (Pierce) pH 7.4) for 2 h at room temperature. 0.1 mm silica glass beads were then added and the cells were disrupted by bead-beating (4 x 45 secs on, 45 secs ice between cycles, 6 m/sec, FastPrep-24 5G (MP Biomedicals) followed by sonication (water sonicator bath) at room temperature for 15 min. The samples were centrifuged ( $2,300 \times g$ , 20 min at 4 °C) and the supernatant collected. Triton X-114 (4% v/v) in PBS was added to the supernatant extract and rocked overnight at 4 °C and insoluble material was removed by centrifuging ( $15,700 \times g$ , 10 min at 4 °C) and the supernatant, removed and stored at 4 °C. For maximum recovery the insoluble pellet was resuspended in 200  $\mu\text{L}$  PBS containing 4% Triton X-114 and rocked overnight at 4 °C. Samples were centrifuged ( $15,700 \times g$ , 10 min at 4 °C) and supernatant removed and pooled. Pooled supernatants were incubated at 37 °C for 15 min and then centrifuged ( $15,700 \times g$ , 10 min at 37 °C). The upper aqueous phase was carefully removed. The proteins in the lower detergent phase were precipitated with 9x volumes of acetone at -20 °C for 18 hours, centrifuged ( $20,200 \times g$ , 10 min, 4 °C), washed twice with ice-cold acetone and resuspended in PBS + 10% glycerol (100  $\mu\text{L}$ ) with sonication (water sonicator bath) at room temperature for 20 min.

### **Proteomic analysis**

Proteins samples (15  $\mu\text{L}$ ) were mixed with 2x SDS loading dye and loaded directly onto the SDS-gel (BioRad Any kD Mini-PROTEAN TGX) and run for 5 min. The gel bands were cut from the gel and prepared for proteomics analysis as described previously.<sup>3</sup> In brief, samples were reduced with 10 mM tris-2(-carboxyethyl)-phosphine (TCEP), alkylated with 40 mM chloroacetamide (CAA) and then in-gel digested with trypsin (2.5 ng/mL) and the peptides extracted with 25% acetonitrile containing 5% formic acid. The extracted peptides were dried under vacuum to a volume of 20  $\mu\text{L}$  and resuspended to a total volume of 50  $\mu\text{L}$  in 2 % acetonitrile, 0.1% trifluoroacetic acid.

### **NanoLC-ESI-MS/MS Analysis**

Protein Mass Spectrometry was performed on a Thermo Orbitrap Fusion (Thermo Scientific) coupled to an Ultimate 3000 RSLCnano HPLC (Dionex) using an Acclaim PepMap  $\mu$ -precolumn cartridge (300  $\mu\text{m}$  i.d. x 5 mm, 5  $\mu\text{m}$ , 100  $\text{\AA}$ ) and an analytical Acclaim PepMap RSLC column (75  $\mu\text{m}$  i.d. x 50 cm, 2  $\mu\text{m}$ , 100  $\text{\AA}$ ,

Thermo Scientific). Mobile phase buffer A was composed of 0.1 % formic acid in H<sub>2</sub>O and mobile phase B was composed of acetonitrile containing 0.1% formic acid. The gradient was programmed as follows: 4 % B increased to 25 % B over 90 min, then further increased to 35 % B over 13 min, followed by 3 min 90 % B with a flow rate of 250 nL/min. Survey scans of peptide precursors from 375 to 1575 *m/z* were performed at 120K resolution (at 200 *m/z*) with a 2x10<sup>5</sup> ion count target. The maximum injection time was set to 150 ms. Tandem MS was performed by isolation at 1.2 Th using the quadrupole, HCD fragmentation with normalised collision energy of 33, and rapid scan MS analysis in the ion trap. The MS<sup>2</sup> ion count target was set to 3x10<sup>3</sup> and maximum injection time was 200 ms. Precursors with charge state 2–6 were selected and sampled for MS<sup>2</sup>. The dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on and instrument was run in top speed mode.

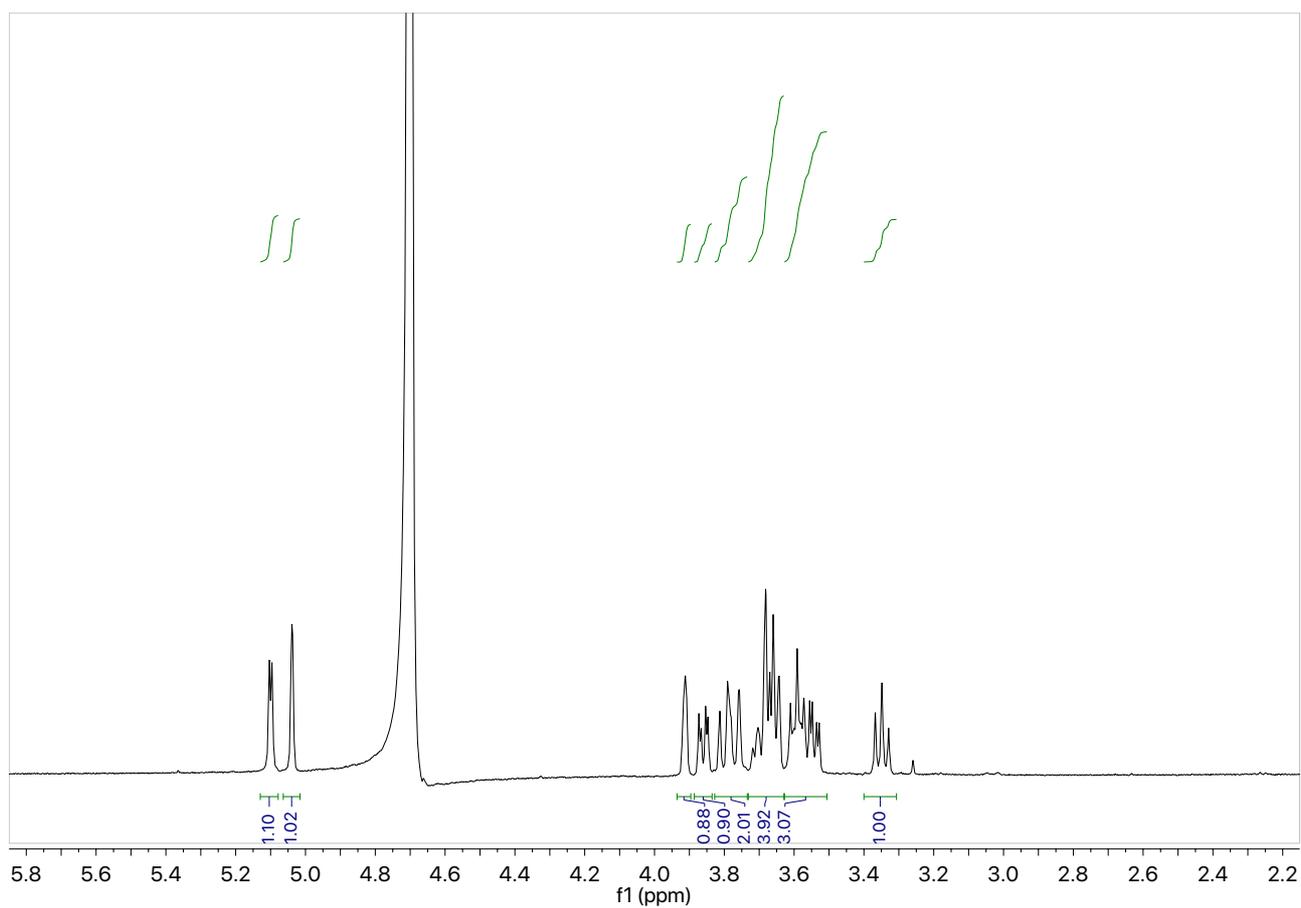
### **Data Analysis**

The raw data were searched using MaxQuant engine (V1.5.5.1)<sup>4</sup> against both the *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* databases and the common contaminant database from MaxQuant.<sup>5</sup> Peptides were generated from a tryptic digestion with up to two missed cleavages, carbamidomethylation of cysteines as fixed modifications, and oxidation of methionines as variable modifications. Precursor mass tolerance was 10 ppm and product ions were searched at 0.8 Da tolerances.

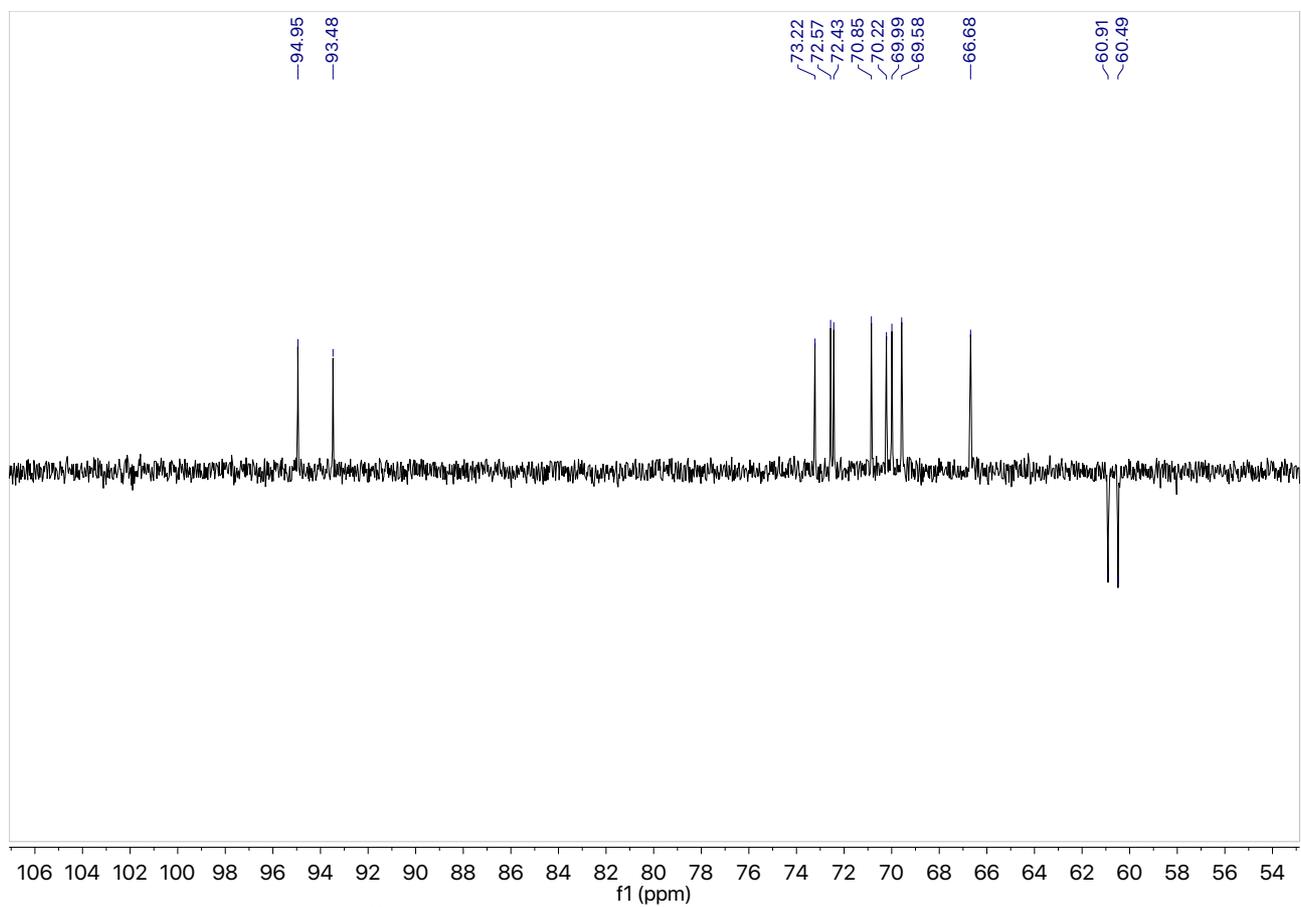
### **Data processing and annotation**

Scaffold (version 4.6.2) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Protein groups with significant total spectra intensity were determined according to the T-Test, using Benjamini-Hochberg multiple test correction and  $p < 0.1$ . Data processing and annotation was performed using the Perseus module of MaxQuant version 1.6.2.2.

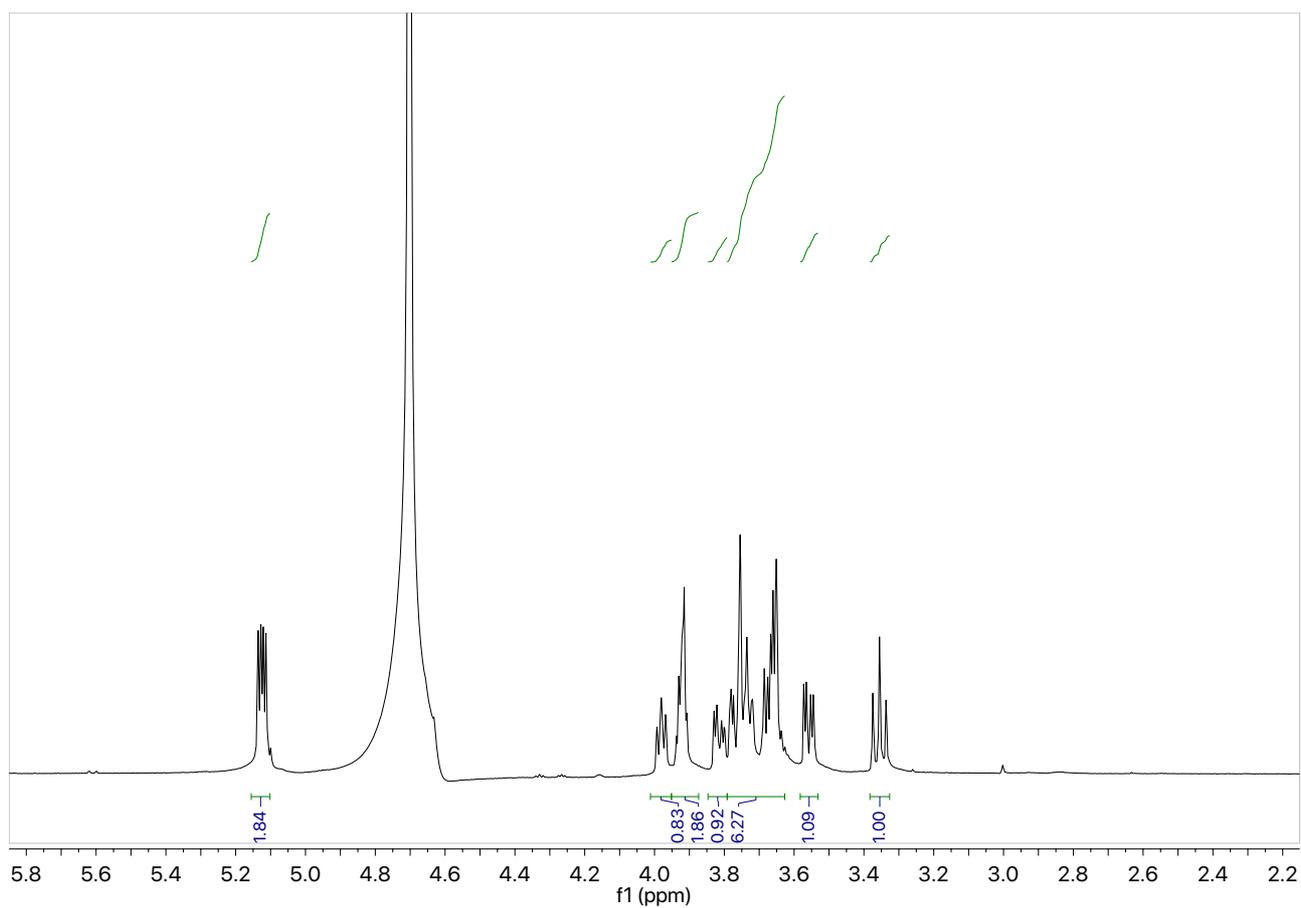
<sup>4</sup> First, we eliminated the reverse and contaminant hits (as defined in MaxQuant) from the MaxQuant output files. Only protein groups identified with at least two uniquely assigned peptide and quantified with a minimum of two ratio counts were used for the analysis. For each experiment, the label free quantification intensity (LFQ) were transformed using the binary logarithm ( $\log_2$ ). Protein groups were considered reproducibly quantified if identified and quantified in at least two replicates, missing LFQ intensity scores were assigned from a normal distribution. Protein groups were assigned a probability value (p-value) using a two-sample Student's T-Test. p-values were subject to a  $-\log_{10}$  transformation. Proteins were considered significant if the  $p\text{-value} < 0.05$  ( $-\log_{10}(p\text{-value}) > 1.30$ ) and had a two-fold change in protein expression ( $\log_2(\text{LFQ difference}) > 1$  or  $< -1$ ). Protein function, product, functional category were assigned based on Mycobrowser (release 3) annotations<sup>6</sup>.



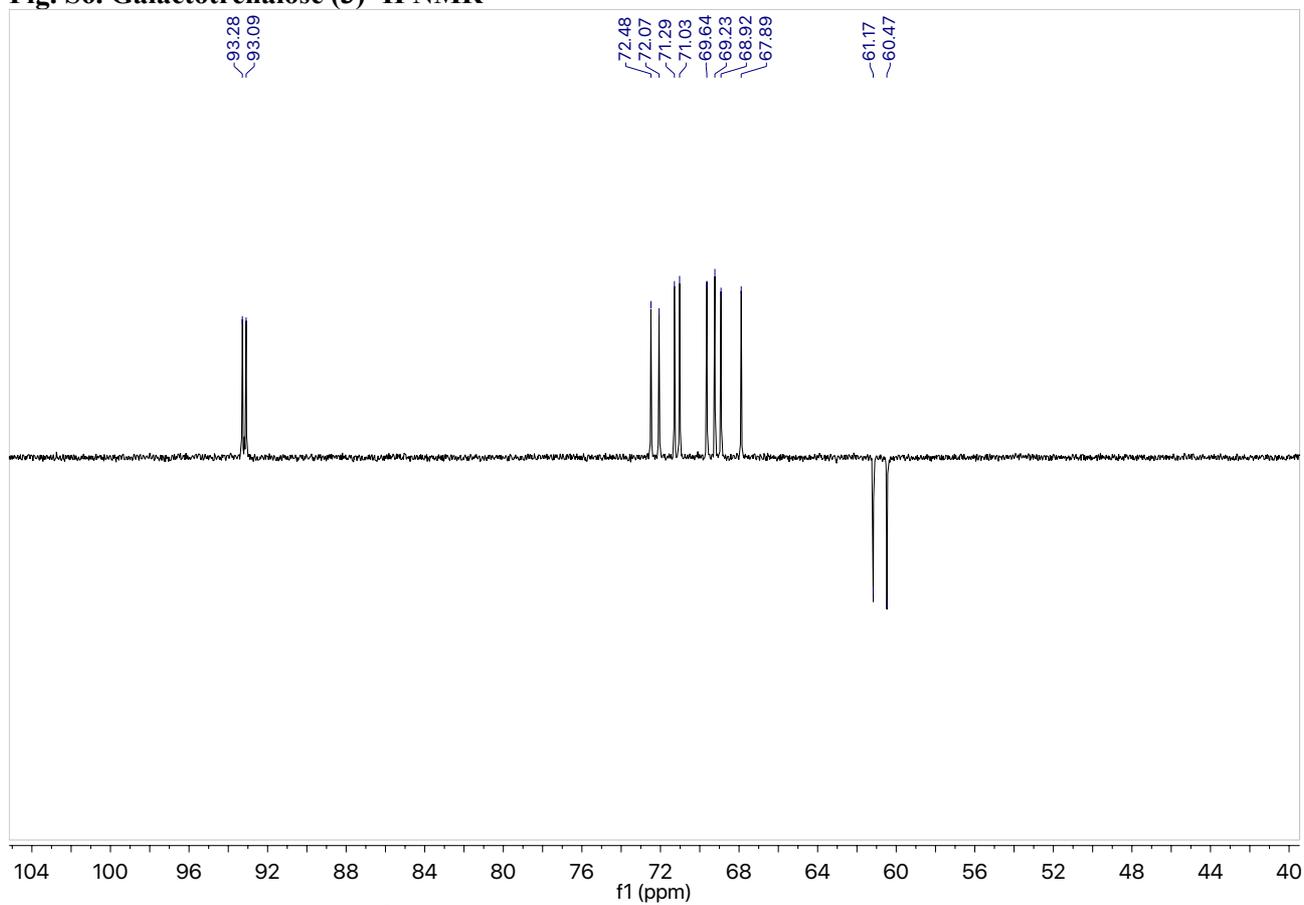
**Fig. S4. Mannotrehalose (2) <sup>1</sup>H NMR**



**Fig. S5. Mannotrehalose (2) <sup>13</sup>C NMR**



**Fig. S6. Galactotrehalose (3)  $^1\text{H}$  NMR**



**Fig. S7. Galactotrehalose (3)  $^{13}\text{C}$  NMR**

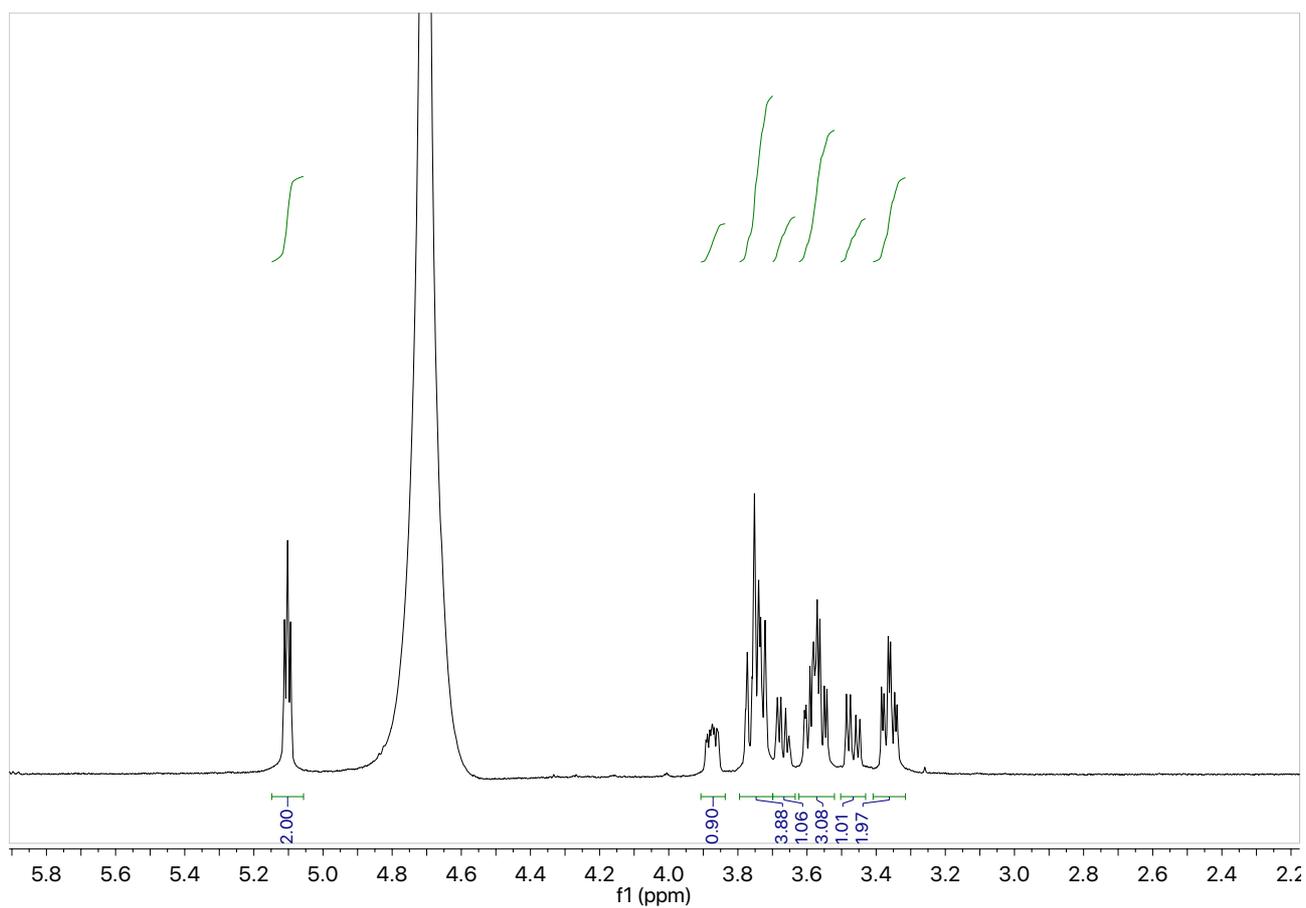


Fig. S8. 6-Azido-trehalose (4) <sup>1</sup>H NMR

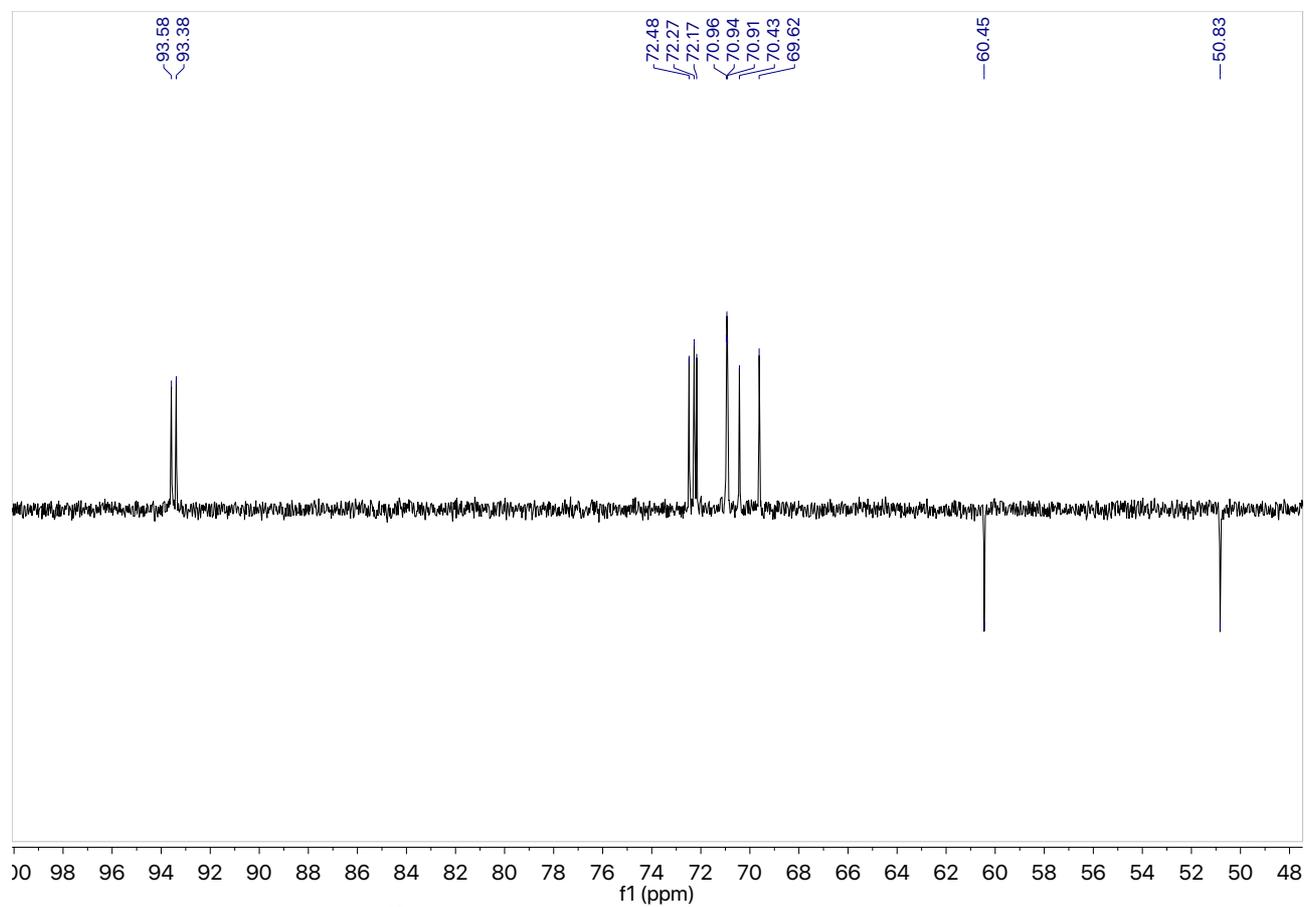
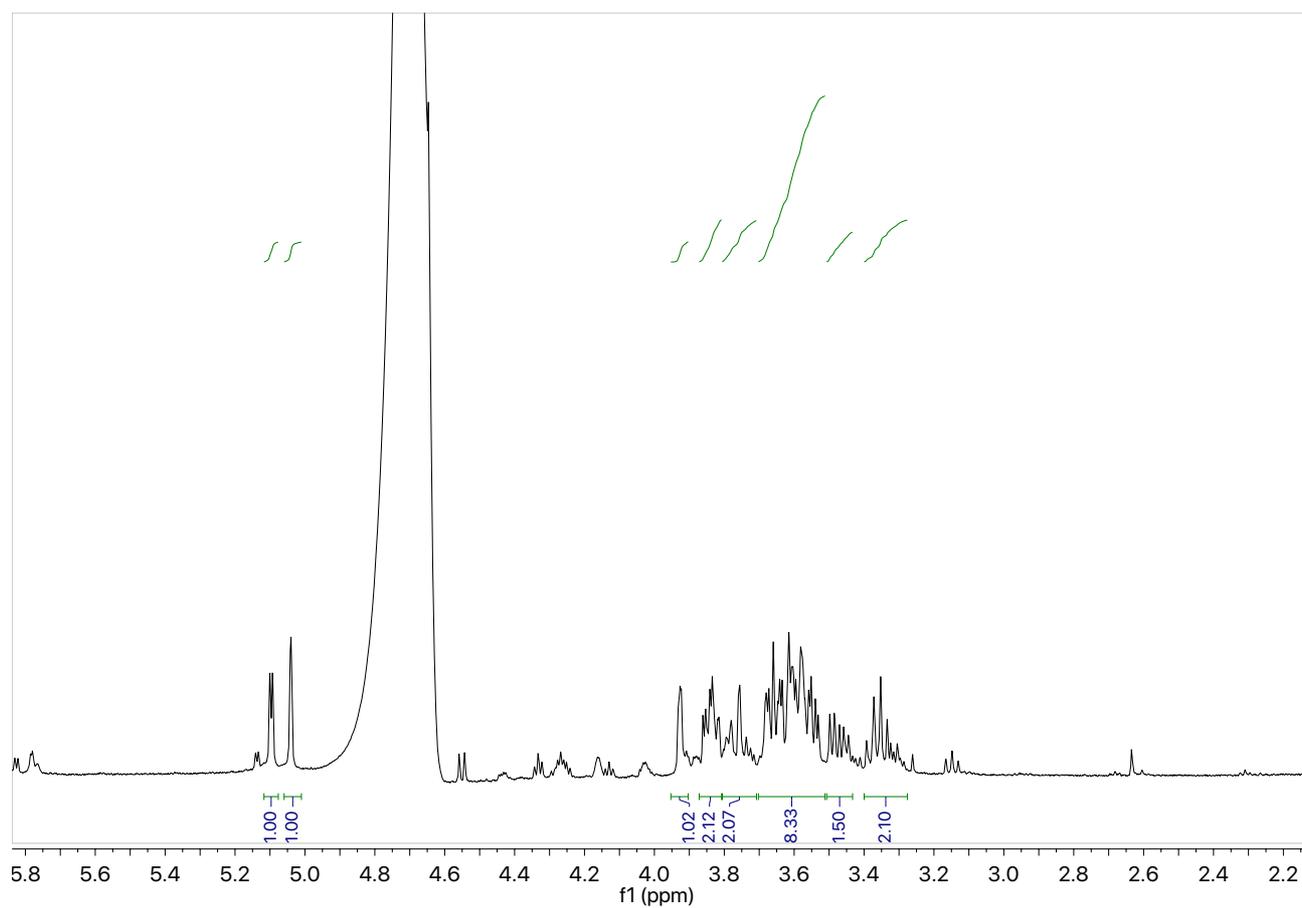
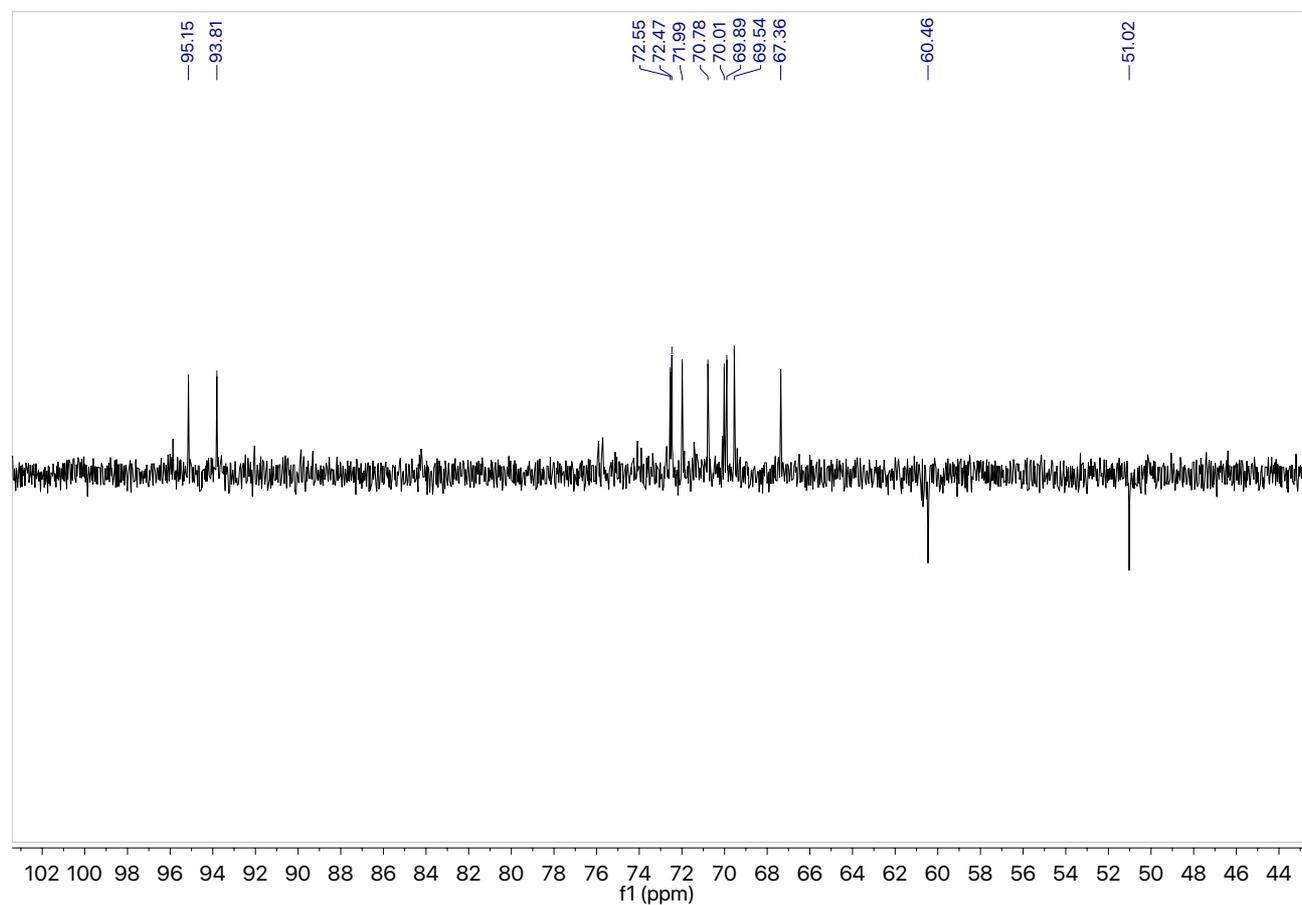


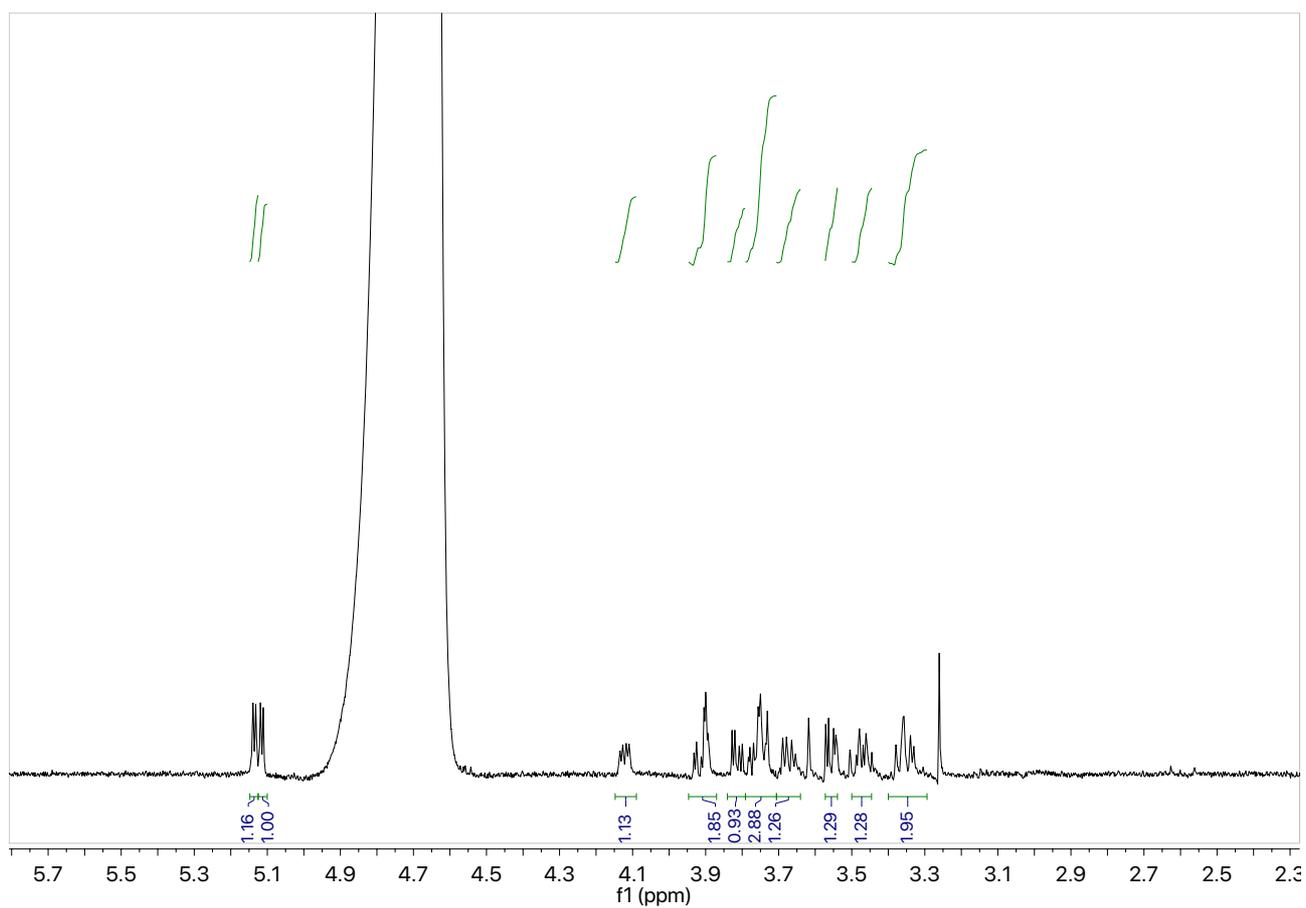
Fig. S9. 6-Azido-trehalose (4) <sup>13</sup>C NMR



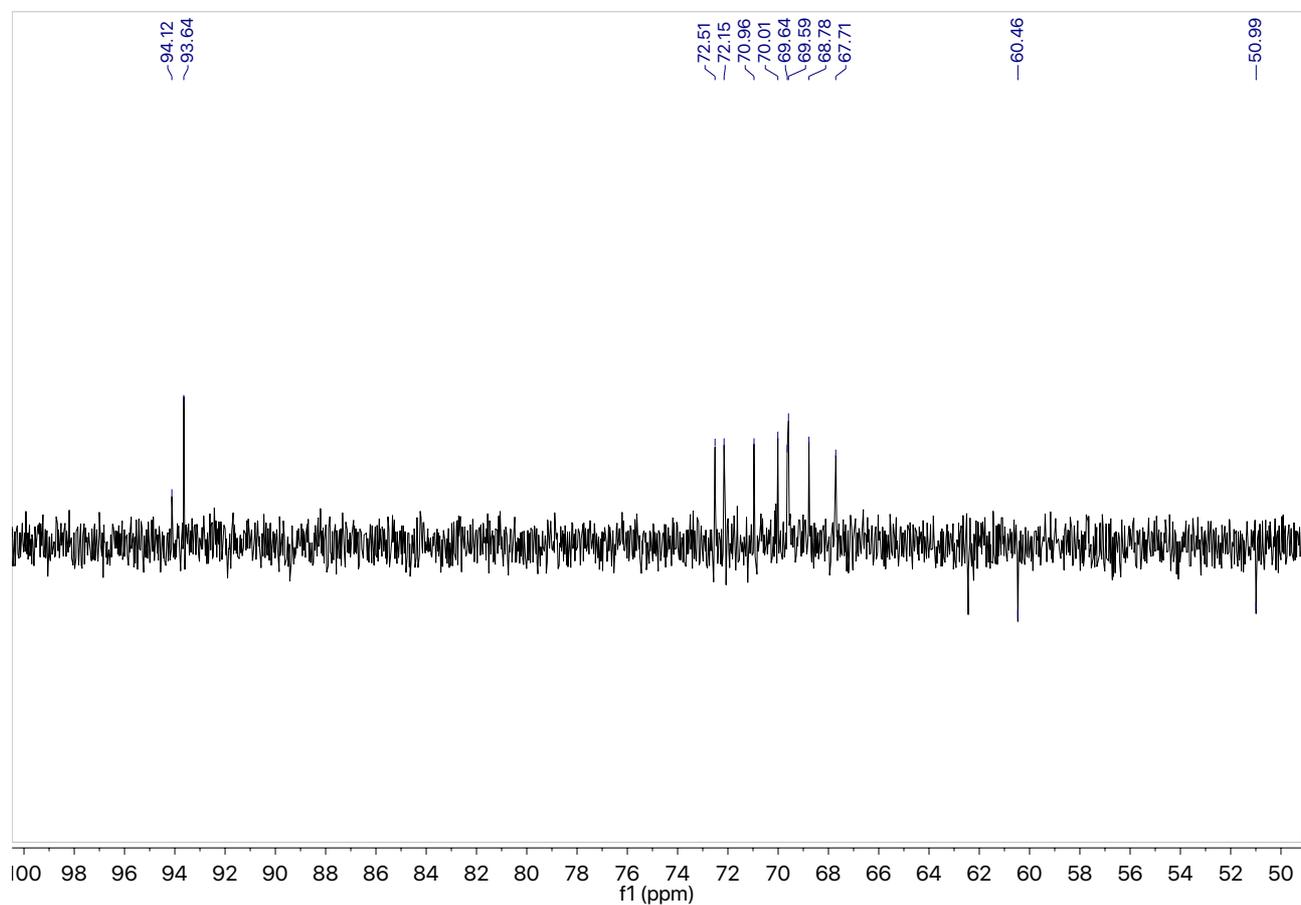
**Fig. S10. 6-Azido-mannotrehalose (5)  $^1\text{H}$  NMR.** Note - impurity peaks are observed at 3.15 ppm, between 4.0 and 4.6 ppm and between 5.15 and 6.0 ppm



**Fig. S11. 6-Azido-mannotrehalose (5)  $^{13}\text{C}$  NMR**



**Fig. S12.** 6-Azido-galactotrehalose (**6**)  $^1\text{H}$  NMR. Note that peaks at 3.26 and 3.62 are impurities.



**Fig. S13.** 6-Azido-galactotrehalose (**6**)  $^{13}\text{C}$  NMR. Note that the peak at 62.4 is an impurity.

## References

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