

Distributable, Metabolic PET Reporting of Tuberculosis

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Supplementary Methods

Unless otherwise noted, all chemicals and solvents were purchased from Sigma-Aldrich (Milwaukee, WI, USA), Fisher Scientific (Hanover Park, IL, USA), Sigma-Aldrich UK, Alfa Aesar, Fisher UK, Carbosynth or Acros and used without further purification. Columns and Sep-Pak cartridges used in this synthesis were obtained from Agilent Technologies (Santa Clara, CA, USA) and Waters (Milford, MA, USA), Phenomenex UK respectively. Sep-Pak was conditioned with 5 mL ethanol. Analytical HPLC analyses for radiochemical work were performed on an Agilent 1200 Series instrument equipped with multi-wavelength detectors. Mass spectra (MS) of decayed [^{18}F]FDT solution were recorded on a 6130 Quadrupole LC/MS, Agilent Technologies instrument equipped with a diode array detector. LC-MS analysis of fluorine-18 labeled trehalose was performed on Agilent 1260 HPLC system coupled to an Advion expression LCMS mass spectrometer with an ESI source. The LC inlet was Agilent 1200 series chromatographic system equipped with 1260 quaternary pump, 1260 Infinity autosampler, 1290 thermostatted column compartment, and radiation detector. Column flow was split (1:4) between the mass spectrometer and the radiation detector. Instrument control and data processing performed using Advion's Mass Express and Quant Express Software.

General Biology Experimental Procedures

All biological equipment and prepared biological solutions, such as media, plastic and glassware, used in the handling of microbial cultures were autoclaved at 121 °C. All biological work was performed in a BASSAIRE Class II Laminar Flow Cabinet. Bacterial cultures were incubated in Innova® 42 incubator Shaker Series. Centrifugation was performed in Avanti Jxn-30 Centrifuge for cell harvesting and post sonicated cell cultures, and Megafuge Centrifuge was used for protein concentration and DNA samples. Micro

centrifugation was performed on Eppendorf Centrifuge 5424 R. Gel electrophoretic analysis was performed on the following equipment: Invitrogen XCell SureLock™ and BioRad Power Pac Basic (200 V used, 50 min.) with using MOPS/MES as buffer.

Preparation of Lysogeny Broth Agar Plates

Lysogeny broth (LB, 6.25 g) and agar (2.5 g) were both added to water (250 mL) and the mixture was autoclaved. After cooled to ~45 °C, a stock solution of appropriate antibiotic was added and the agar was poured into plates and allowed to settle. Stored at 4 °C.

Transformation

Plasmid stock (1 µL, 10 ng) and cell stock (20 µL) mixed in 14 mL polyene tube and incubated on ice for 30 min, the mixture was then heat shocked to 42 °C for 45 s then incubated on ice for 2 min. Super optimal broth with catabolic repression (SOC) medium (200 µL) was added and the mixture was incubated at 37 °C for 1h. The mixture was plated on agar plates with the appropriate antibiotic (2 plates, one with 100 µL one with 25 µL). Plates were then incubated overnight (37 °C).

DNA Preparation

A single colony was picked and placed into 5 mL of lysogeny broth (LB) with the appropriate antibiotic. The culture was shaken at 37 °C overnight. Cells were pelleted by centrifugation (4700 rpm, 20 min, 4 °C) then DNA preparation carried out according to the instructions provided with the QIAGEN QIAprep spin miniprep kit. DNA concentration was determined by absorption on a Nanodrop and Sanger sequencing performed by Source Bioscience. Sequence analysis was performed by using source bio edit.

SDS-PAGE

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check the purity of all proteins. These were carried out using 4 – 12% bis-Tris polyacrylamide gels with either MOPS or MES buffer. To the protein sample (8 μ L) was added 2 \times SDS loading buffer (3 μ L 80 mM Tris-HCl buffer pH 6.8, 8% v/v glycerol, 40 μ M bromophenol blue, 80 mM SDS, 2% β -mercapethanol). The solution was then heated to 95 $^{\circ}$ C for 10 min. The sample was then applied to a well in the gel. The gel was then run at 200 V for 1 h and then visualised by the addition of Coomassie InstantBlue dye (Gentauro) and gentle stirring for 2 h at room temperature.

Western Blots

SDS-PAGE was done as before, but using a Pre-Stained protein ladder and the gel was not stained with InstantBlue dye. Instead, the gel was electroblotted onto a membrane for 7 min using an iBlot machine. The membrane was then washed with 50 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.6 (TBS-T + 5% BSA w/v , 45 mL) for 1 h. The membrane was further washed with TBS-T + 5% BSA w/v, 45 mL and monoclonal anti-polyHistidine-alkaline phosphatase antibody produced in mouse (Sigma, 16 μ L). The membrane was then rocked for 4 h at room temperature. After this time, the solution was removed and the membrane washed with TBST buffer (3 \times 20 mL). BCIP/NBT alkaline phosphate substrate (Sigma Aldrich, 4 mL) was added and the membrane was gently rocked until protein bands could be seen (~15 min). The membrane was then washed with water.

Protein Mass Spectrometry

Various machines used for protein LC-MS. In all cases solvent A was water and solvent B was acetonitrile with both containing 0.1% formic acid.

Set-up 1: Waters LCT Classic coupled to a Shimadzu 20 Series HPLC using a Thermo Proswift (250 x 4.6 mm x 5 μm) column. Electrospray source parameters were as follows: capillary voltage 3000 V; cone voltage 25 V. Spectra were calibrated to at least 17 matched peaks of the multiply charged ion series of equine myoglobin run under equivalent conditions. The gradient program is shown below; a flow rate of 0.4 ml/min was used throughout (see also Supplementary Table S3).

Set-up 2: Waters Xevo G2-QS QToF MS coupled to a Water Acquity UPLC using a Thermo Proswift (250 x 4.6 mm x 5 μm) column. Electrospray source parameters were as follows: capillary voltage 3000 V; cone voltage 20 V. Spectra were calibrated through use of an internal lock-spray. The gradient program is shown below; a flow rate of 0.3 ml/min was used throughout (see also Supplementary Table S4).

Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 l hr⁻¹. Spectra were calibrated using a calibration curve constructed from a minimum of 16 matched peaks from the multiply charged ion series of equine myoglobin obtained at a cone voltage of 35 V. Data was processed using MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.

Circular Dichroism (CD) Spectroscopy

For CD measurement, protein was buffer exchanged into phosphate buffer (10 mM potassium phosphate, pH 7.5). Measurements were made at room temperature using a Chirascan CD-spectrometer (Applied Photophysics) using 0.1 cm quartz cuvette. Background readings were taken with using 0.2 mL of buffer only, followed by protein samples measured at

concentrations of 0.2 mg/mL. Scans were run using 0.5 s per time point and 0.5 nm increments, scanning from 185 to 280 nm. Data reported is the mean average of 3 results.

Liquid Chromatography-mass spectrometry for Small Molecule Analysis

Liquid chromatography–mass spectrometry (LC–MS) for quantification of trehalose and glucose was performed on the LC-MS Quattro instrument coupled with agilent technologies HPLC system, using a TOSOH TSKgel Amide-80 column (4.6 mm x 250 mm, 5 μ M, 80 Å). Solvent A (CH₃CN) and (H₂O) with a volume ratio of 70:30 were used as the isocratic mobile phase at a flow rate of 1.0 mL / min. The MS file was programme as 20 min for each sample. The electrospray source was operated with a capillary voltage of 3.0 kV and a cone voltage of 20 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 500 Lh⁻¹. The data was acquired by single ion recording (SIR) of m/z 341 for trehalose and m/z 179 for glucose in negative mode and processed using MassLynx software (v. 4.1 from Waters) according to the manufacturer’s instructions. See Supplementary Table S9 for the LC-MS methods employed in the detection of FDT.

High Performance Liquid Chromatography (HPLC) for Enzyme Reaction Analysis

All of the enzyme reactions were analysed by HPLC for UDP-glucose consumption and UDP release. For the analysis, few different stationary and mobile phases were used to optimise the separation between UDP and UDP-glucose (summarised in Supplementary Table S5).

Method 3 was selected as a method of choice because it gave the best separation between the two phosphate sugars.

Expression, Purification and Characterisation of OtsA *E. coli*

pET-22b(+) C-Terminal-His vector containing gene for OtsA was transformed into BL21 (DE3) cells using standard transformation protocol. The transformed cells were used to inoculate an overnight culture in 100 mL of freshly prepared LB medium containing 100 μ L of Ampicillin stock solution. The culture was incubated overnight at 37 °C. The overnight culture was used to isolate plasmid DNA using the standard QIAGEN miniprep kit protocol. The gene construct was confirmed by sequencing.

In a typical expression experiment performed on a 800 mL LB medium scale, transformed cells were grown overnight at 37 °C in 100 mL of freshly prepared LB containing 100 μ L of Ampicillin stock solution (100 mg/mL). 4 mL of overnight culture was used to inoculate 800 mL of freshly prepared LB media containing 800 μ L of Amp stock solution. The culture was incubated at 30 °C with shaking. OD₆₀₀ was measured from time to time. Once the OD ~ 0.45, protein expression was induced by adding 4 mL of 1 M IPTG solution to a final concentration of 0.5 M. The culture was left to grow overnight at 30 °C for 18 hours. The culture was harvested by centrifugation (8000 rpm, 10 minutes, 4 °C). Cell pellet was suspended in binding buffer (50 mM Hepes, 200 mM NaCl, 10 mM imidazole, pH 7.5) and one tablet of Complete protease inhibitor cocktail (Roche), DNAase and lysozyme was added. The mixture was stirred vigorously at 4 °C for 2 hours. The cells were lysed via sonication. The suspension was then centrifuged (14000 rpm, 30 min) to remove cell debris. The supernatant was then filtered using a 0.45 μ m syringe filter. The filtered supernatant was applied to a pre-equilibrated (using binding Buffer) GE Life Sciences 5mL HisTrap Ni-NTA column. Purification was done at 4 °C. The column was washed by 15 column volumes of binding buffer, followed by a linear gradient to 100 % Elution Buffer (50 mM Hepes, 200 mM NaCl, 500 mM Imidazole, pH 7.5) over 16 column volumes. Eluted protein fraction were analysed using SDS-PAGE analysis.

Expression, Purification and Characterisation of His-MBP-OtsB from *E. coli*

In a routine restriction digestion reaction for vectors, 2 μL of each restriction enzyme (NdeI & XhoI), 3 μL of NEB buffer 4 (10x), 1 μg of vector (pDB-His-MBP) were taken in an eppendorf tube. dd H₂O was added to reaction mixture to make reaction volume 30 μL . The reaction mixture was incubated at 37 °C for 4 h. For insert, similar reaction was set-up with 3 μL of OtsB gene in pET-29b(+) vector. Digested vector and insert was loaded onto 1% Agarose gel. The gel was run at 110 V for 40 minutes.

Restriction-digested products were extracted from agarose gel using the standard QIAquick gel extraction kit protocol. Under UV transilluminator, gel fragments containing restriction-digested products were excised using a clean, sharp scalpel. Gel were transferred to eppendorf tubes, weight of gel was taken. 3 volumes of QG buffer were added to 1 volume of gel. The sample was incubated at 50 °C for 10 minutes with occasional vortexing. 1 gel volume of isopropanol was added and was mixed by inverting the eppendorf tube 4-6 times. The sample was decanted into a spin column and was centrifuged for 1 minute. The flow through was discarded. 500 μL of QG buffer was added to the column and centrifuged for 1 minute. The supernatant was discarded and 750 μL of buffer PE was added to the column. The spin column was centrifuged for 1 minute. The supernatant was discarded, to remove the residual wash buffer; column was centrifuged further for 1 minute. The spin column was placed in a new Eppendorf tube and 50 μL of dd H₂O was placed at the centre of column. The column was left to stand for 4-5 minutes, after which it was centrifuged for 1 minute. The DNA was stored at -20 °C for further manipulation. Concentration of products was calculated using Nanodrop.

For ligation, 50 ng of vector was combined with a 3-fold molar excess of PCR product. The volume was adjusted to 20 μL with dd H₂O. 2 μL of 10 x T4 ligation buffer was added and

mixed thoroughly. 1 μL of Quick T4 DNA Ligase was added and mixed thoroughly. Reaction mixture was centrifuged briefly and was incubated at 25 °C for 5 minutes. The ligated product was stored at - 20 °C. Transformation of the ligated product was done in XL10 Gold competent cells through standard transformation protocol using 2 μL of ligated reaction mixture. Transformed cells were plated on LB-Agar/Kan plates and incubated at 37 °C overnight.

Single colonies of transformed cell were used to inoculate overnight culture. Plasmid DNA was isolated from the overnight culture using standard miniprep protocol. The successful sub-cloning was verified by sequencing.

pDB-His-MBP vector containing gene for OtsB was transformed into BL21 (DE3) cells using standard transformation protocol. A single transformed cell was used to inoculate a 100 mL starter culture of freshly prepared LB medium containing 100 μL of Kanamycin stock solution (50 mg/mL). The culture was incubated at 30 °C for 16 hours. 8 mL of starter culture was used to inoculate 800 mL of LB medium containing 800 μL of Kanamycin stock solution. The culture was incubated at 37 °C with shaking. OD600 was measured from time to time. Once OD600 reaches close to 0.6. Expression was induced by adding 800 μL of 1 M IPTG solution. The culture was incubated at 30 °C with shaking for 16 hours.

The culture was harvested by centrifugation (8000 rpm, 10 minutes, 4 °C). Cell pellet was suspended in binding buffer (50 mM Tris, 200 mM NaCl, 20 mM Imidazole, pH 7.5) containing Complete protease inhibitor cocktail (Roche), DNAase and lysozyme. The mixture was stirred vigorously at 4 °C for 2 hours. The cells were lysed via sonication using program P9 consisting of 0.5 sec on, 0.5 sec off pulses for 30 sec followed by 1 minute of cooling at 40% amplitude. The sonication was done for 5 cycles. The suspension was centrifuged (20000 rpm, 40 min) to remove cell debris. The supernatant was then filtered using a 0.45 μm syringe filter. The filtered supernatant was applied to a pre-equilibrated (Binding Buffer) GE

Life Sciences 5ml HisTrap HP column. Purification was done at 4 °C. The column was washed by 10 column volumes of binding buffer, followed by a linear gradient to 100 % Elution Buffer (50 mM Tris, 200 mM NaCl, 500 mM imidazole, pH 7.5) over 15 column volumes. Purification was done at a flow rate of 5 mL/min. Eluted protein fraction were analysed using SDS-PAGE analysis. Buffer exchange was done using dialysis tubing with MWCO 10,000 Da. Fractions containing the ca. 72 KDa protein were pooled and dialyzed thrice against 4 liters of 50 mM Tris, pH 7.5 and stored at 4 °C. Protein were concentrated using Vivaspin with MWCO 30,000 Da to a final concentration of 12.55 mg/mL. The protein was further stored at -20 °C and characterised by SDS-PAGE.

Expression, Purification and Characterisation of TreT *P. horikoshii* Enzyme

The DNA plasmid from group plasmid bank was verified by sequencing from source bio-science. The plasmid encoded a C-terminal 6 x Histidine tag (His-tag) on the protein. The plasmid was transformed into *E.coli* (BL21 DE3) cells and plated on LB agar containing 50 mg/mL kanamycin. One of the resulting grown colony was inoculated into 10 mL LB media containing 10 µL of kanamycin (50 mg/mL) solution and left incubating overnight (37 °C, 200 rpm).

To express the TreT protein, 10 mL grown culture was inoculated into 1 L of LB media containing 1 mL of kanamycin solution (50 mg/mL) and left incubating (37 °C, 200 rpm). OD₆₀₀ was measured every one hour and once the inoculated culture reached the mid-log phase, it was induced with 1 mL of IPTG solution (final concentration = 1 mM) and grown in shaking incubator overnight (37 °C, 200 rpm).

Cells were then harvested by centrifugation (4700 rpm, 20 min, 4 °C) and the pellet was resuspended in 30 mL lysis buffer (300 mM NaCl, 50 mM NaH₂PO₄, 20 mM imidazole). One

protein inhibitor tablet, 25 mg lysozyme and 5 mg of DNase was also added into the lysate and lysis reaction was carried out on rotating platform (60 min, 4 °C). Lysed cells were sonicated (5 x 30 s, 30 s pulse on, 60 s pulse off, 75% amplitude). Sonicated cells were centrifuged (21000 x g, 20 min, 4 °C) to clarify the lysate. Supernatant was then filtered (0.45 µm and 0.2 µm filter). Next supernatant was loaded on a 50 mL FPLC loop and purified by Ni-NTA chromatography using 5 mL His-trap HP column and eluted with 15 column volumes of elution buffer (300 mM NaCl, 50 mM NaH₂PO₄, 250 mM imidazole). Protein samples were concentrated using Vivaspin at 10 kDa MWCO (Sartorius). Elution buffer was exchanged for 300 mM NaCl, 50 mM HEPES, pH 7.5. Protein samples were then characterised by SDS-PAGE to verify protein purity and LC-MS to confirm the mass of the protein.

Expression, Purification and Characterisation of TreT *T. tenax* Enzyme

2 x 1 L cultures of TreT *T. tenax* protein were expressed in BL21 (DE3) cells. Expression was carried out by inoculating 10 mL cultured BL21 (DE3) transformed with plasmid DNA into 1 L of LB media. 1 mL of Kanamycin sulphate solution (50 mg / mL) was used as the resistance antibiotic. Both the flasks were shaken at 200 rpm and 37 °C for 3 hours when the OD₆₀₀ value reached to 0.6. At this stage protein induction was carried out by using 1.0 mL of IPTG solution (final concentration = 1 mM), post induction both flasks were then kept overnight in the shaking incubator. Cells were then harvested by centrifugation (4700 rpm, 20 min, 4 °C), and the pellet was resuspended in 30 mL lysis buffer (300 mM NaCl, 50 mM NaH₂PO₄, 20 mM imidazole). One protein inhibitor tablet, 25 mg lysozyme and 5 mg of DNase was also added into the lysate and lysis reaction was carried out on rotating platform (60 min, 4 °C). Lysed cells were sonicated (5 x 30 s, 30 s pulse on, 60 s pulse off, 75% amplitude). Sonicated cells were centrifuged (21000 x g, 20 min, 4 °C) to clarify the lysate.

Supernatant was then filtered (0.45 μm and 0.2 μm filter). Next supernatant was loaded on a 50 mL FPLC loop and purified by Ni-NTA chromatography using 5 mL His-trap HP column and eluted with 15 column volumes of elution buffer (300 mM NaCl, 50 mM NaH_2PO_4 , 250 mM imidazole).

FPLC fractions were then analysed by SDS-PAGE. Fraction containing potential protein were concentrated using Vivaspin at 10 kDa MWCO (Sartorius) and centrifuging at 8000 x g.

Protein concentration was determined by nanodrop and characterisation was done with mass spectrometry and SDS-PAGE.

Plasmid Design and Trial Expression of OtsAB Fusion Enzyme

N-terminus His-tagged OtsAB fusion protein plasmid DNA was transformed into BL21 (DE3) cells. The grown colonies were inoculated into the LB media (10 mL) containing 10 μL of ampicillin (100 mg/mL) solution incubated overnight in the shaking incubator (37 $^\circ\text{C}$, 200 rpm). Small scale expression trial was carried out by pipetting 250 μL of grown culture in 20 mL of LB media in 6 different 50 mL falcon tube. 20 μL of ampicillin solution was also added. Tube 1 & 2 were used as control (0.4 OD_{600} and 0.6 OD_{600}); tube 3 & 4 were induced with 0.5 mM and 1 mM IPTG solution when OD_{600} value was 0.4 and 0.6 respectively. A similar process was repeated for tube 5 & 6. 1 mL of sample was taken from each tube at 0 min, before adding IPTG solution, 4 h, 16 h, 24 h and 48 h post IPTG induction.

Cells were then harvested from liquid culture by centrifugation (10,000 x g, 10 min, 4 $^\circ\text{C}$). The cell solution was decanted and the pellet was allowed to drain completely. 100 μL of bugbuster reagent was added into each of the sample and the cell pellet was completely re-suspended by vortexing slowly. Cells were then incubated at a shaking platform for 20 min at room temperature. Insoluble cell debris was removed by centrifugation (16000 x g, 20 min, 4 $^\circ\text{C}$). Supernatant was transferred into a fresh tube and analysed by SDS-PAGE.

Large Scale Expression of OtsAB Fusion Protein

N-terminus His-tagged OtsAB fusion protein plasmid DNA (2 μ L) was transformed into BL21 (DE3) cells (20 μ L). The grown colonies were inoculated into the LB media (10 mL) containing 10 μ L of ampicillin solution (100 mg/mL) incubated overnight in the shaking incubator (37 °C, 200 rpm). Large scale expression was carried out by inoculating 10 mL of grown culture in 1000 mL of LB media in 2 different 2L conical flasks. 1000 μ L of ampicillin solution (100 mg/mL) was also added. Both flasks were then incubated in the shaking incubator (37 °C, 200 rpm) until the OD₆₀₀ reached 0.4. Cells were then incubated at 15 °C for further 30 min and then induced by adding IPTG solution (1 mL, 1 mM) and incubated for further 24 h at 15 °C.

After 24 h cells were harvested by centrifugation (9000 rpm, 20 min, 4 °C) and lysed with lysis buffer (50 mM Tris, 50 mM imidazole and 150 mM NaCl, pH 7.5) for 30 min at room temperature. Lysis was carried out by adding lysozyme (25 mg), DNase (5 mg) and 1 protein inhibitor tablet. Post lysis cells were sonicated (60% amplitude, 6 cycles of 30 s pulse on, 60 s off). Sonicated cells were then centrifuged to get rid of cell debris (20000 rpm, 20 min, 4 °C) and filtered through 0.45 μ m and 22 μ m filter and loaded onto FPLC loop for purification using His-trap HP 5 mL column as stationary phase and eluted with 15 column volumes of elution buffer (50 mM Tris, 150 mM NaCl, 500 mM imidazole).

FPLC fractions were then visualised by SDS-PAGE method and fraction containing potential protein were concentrated through Vivaspin (5 mL, 30 kDa MWCO) by centrifugation (5000 x g, 15 min, 4 °C). Protein samples were then characterised by LC-MS.

Enzymatic Reaction of OtsAB fusion Protein

To test the protein activity enzymatic reaction was carried out. 20 μL Glucose-6-phosphate (20 mM); 20 μL of UDP-glucose (20 mM), 1.25 μL of manganese chloride (2.5 mM) and 20 μL of enzyme (0.23 μM) were added into 1.5 mL Eppendorf tube. The total reaction volume was adjusted to 100 μL by adding Tris/NaCl buffer (50 mM Tris; 150 mM NaCl, pH 7.5).

Reaction was started by adding the enzyme solution and stopped by placing on ice and quenched by adding 20 μL acetonitrile solution. Reaction was carried out on the thermoshaker (37 $^{\circ}\text{C}$, 700 rpm) and continued for 24 h.

Detection of Trehalose by LC-MS for TreT and OtsAB Fusion enzyme

In order to detect the trehalose LC-MS method was used as described in the general experimental condition. Standard solutions for glucose and trehalose were analysed along with the samples. For TreT reaction UDP-glucose was used in excess and 8 mM glucose was used as a substrate. Enzyme concentration, metal ion concentration and buffer amounts were kept the same as in the case UDP-Glucose and UDP reaction.

For OtsAB fusion reaction, total reaction volume was 100 μL . In 1.5 mL Eppendorf tube were added: glucose-6-phosphate 2 μL (10 mM), MnCl_2 1.25 μL (2.5 mM) and 0.23 μM enzyme was used. UDP-Glucose concentration was maintained at 50 μL (50 mM). Reaction was made up to 100 μL by adding Tris/HCl buffer solution (50 mM, pH 7.5).

Reaction Comparison between TreT homologues

To demonstrate the yield of FDT from TreT enzyme reactions, we tested the reaction for both homologues. Equimolar amount of starting substrate and enzyme concentration was used (see

Supplementary Table S6). All the other parameters and reagents concentration was kept constant so that yield for FDT formation from each enzyme system can be compared. In summary reaction volume was maintained to 500 μL and HEPES buffer (50 mM HEPES, pH 7.5, 200 mM NaCl and 10 mM MgCl_2) was used to make up the required volume. Reactions were initiated by adding the enzyme and then incubated at 37 $^\circ\text{C}$, 700 rpm on the thermoshaker. 150 μL of reaction samples were taken after 30 min, 60 min and 120 min respectively from each reaction mixture. Reactions were stopped by incubating the reaction mixture at 100 $^\circ\text{C}$ for 5 min and further diluted to 500 μL with D_2O solution, centrifuged and stored for the analysis. Sample analysis was carried out using 500 MHz NMR spectrometer (AVB500) and ^{19}F -NMR signal for the formation of FDT were sought as compared to FDG peaks in all the reaction mixtures.

Evaluation of Reaction Reversibility with TreT *P. horikoshii*

To test the reversibility of TreT *P. horikoshii*, we set-up small scale reactions from 1-10 mM of ^{19}F FDT with fixed UDP concentration of 20 mM. In 1.5 mL Eppendorf tube were added the following: UDP 20 mM, ^{19}F FDT 1 mM, 2 mM, 5 mM and 10 mM with a further 1 mM control reaction. Reaction volume was made up to 100 μL with HEPES buffer solution (50 mM HEPES, 200 mM NaCl, 10 mM MgCl_2 , pH 7.5). Reactions were initiated by adding TreT enzyme and incubated at 37 $^\circ\text{C}$ for 2 h. A further reaction with higher amount of UDP substrate (80 mM) and ^{19}F FDT (20 mM) was also set-up as above. Reactions were analysed by ^{19}F -NMR spectroscopy.

Enzyme production scale-up in Sf9 cells expression

For higher level expression of OtsA and OtsB proteins, the Bac-to-Bac® Baculovirus Expression System was selected. The original coding sequence of *E. coli* OtsA (CAA48913.1) and OtsB (CAA48912.1) was optimized according to *S. frugiperda* MNPV codon usage. The DNA fragment was then cloned into the *EcoRV* restriction enzyme site of the pUC57 vector. Then the plasmids pUC57-OtsA and pUC57-OtsB and pFastBac Dual vector were digested with *BamHI* and *NotI*, respectively, and ligated to create the recombinant plasmids of OtsA (pFastBac-OtsA) and OtsB (pFastBac-OtsB). The bacmid DNAs of OtsA (Bacmid-OtsA) and OtsB (Bacmid-OtsB) were generated by transformation of the recombinant plasmids pFastBac-OtsA and pFastBac-OtsB into the DH10Bac™ *E. coli*, respectively. Correct insertions of the OtsA and OtsB genes in the bacmid DNA were confirmed by PCR analysis using pUC/M13 standard primers: 5-CCCAGTCACGACGTTGTAAAACG-3 (forward) and 5-AGCGGATAACAATTTACACACAGG-3 (reverse).

Based on the bacmid DNAs, the recombinant baculovirus of OtsA and OtsB were made by transfection of Sf9 cells. Culture and preparation of Sf9 cells were performed according to the protocol provided by manufacturer. A Sf9 cell stock with a viability >95% and a density of $1.5 \times 10^6 - 2.5 \times 10^6$ cells/mL was prepared before proceeding to transfection.

Experimental procedures for generating P1 baculovirus for OtsA and OtsB

The P1 baculovirus stocks of OtsA and OtsB were generated using Sf9 cells in 6-well culture plates. Briefly, 2 ml of Sf9 cells were added to each well of two 6-well plates with the cell number of 8×10^5 in each well. Cells were allowed to attach for 15 minutes at room temperature. Transfectin II was used as the transfection reagent and the transfection experiments were performed according to the protocol provided by manufacturer. Different

amounts of bacmid DNAs of OtsA and OtsB were used. After transfection, the Sf9 cells were further cultured at 27 °C for 3 days, then the P1 baculovirus (in supernatant) was harvested by centrifugation. The P1 baculovirus were found in the culture medium and the Sf9 cell pellets were used to check protein expression. Fetal bovine serum (FBS) was then added to each P1 baculovirus solution to reach a final concentration of 2%. The P1 baculovirus stock solutions were then stored at 4 °C, protected from light.

Amplification of P1 baculovirus of OtsA and OtsB: In order to generate baculovirus stock for large scale protein expression and to increase virus titer, P2 and P3 baculovirus stocks of OtsA and OtsB were prepared from their P1 baculovirus. Briefly, a total of 150 mL of suspended Sf9 cells with a cell density of 2.0×10^6 cells/mL was infected by P1 baculovirus of OtsA or OtsB, respectively. The 150 mL infection culture composition was: 1% P1 baculovirus of OtsA or OtsB, 1% FBS, 1% ethanol, Sf9 cells with a density of 2.0×10^6 cells/mL. After infection at 27 °C with shaking (150 rpm) for 72 hours, the P2 baculovirus (in culture medium) was harvested by centrifugation at 1100 rpm for 10 minutes at room temperature. FBS was added to a final concentration of 2%, then the solution was filtered by syringe filter (0.45 μ m) and stored at 4 °C, protected from light. Similar infection procedures were performed to prepare P3 baculovirus, except using P2 baculovirus stock solution for cell infection.

Expression and Purification of C-terminally tagged, recombinant OtsA

Insect cells were grown in serum-free medium to a density of 2×10^6 cells/mL and a cell viability of greater than 95%. The cell culture (1 L) was transfected with the viral stock, 2.8×10^6 cells/mL. At 72 h post-infection, the cells were harvested by centrifugation. After freeze/thaw cycle, the pellet was resuspended in lysis buffer (100 mM HEPES, 200 mM NaCl, 10 mM imidazole, 10 mM MgCl₂, 1 mM β ME). DNase 3 mg and 1x Complete®

protease inhibitor cocktail was also added into the protein pellet and lysed using a homogenizer. Cell debris was removed by centrifugation and the cell free extract passed through a 0.45 μm membrane filter. The filtrate was applied to a Ni-NTA column equilibrated in lysis buffer. After removal of the cell debris by centrifugation, the protein of interest was purified with a linear gradient of 10–300 mM imidazole in 100 mM HEPES, 200 mM NaCl, 10 mM MgCl_2 , 1 mM βME , pH 7.5, using Ni-NTA. Protein samples were exchanged into 100 mM Hepes, 150 mM NaCl, 10 mM MgCl_2 , pH 7.5, using a Hiload™ 26/60 Superdex™ 200 desalting column. The sample was passed through an Acrodisc Mustang E membrane (Pall #MSTG25E3) to reduce endotoxin to 1.9 EU/mL. After the addition of 10% glycerol, the aliquots containing the recombinant enzyme were flash frozen, and stored at $-80\text{ }^\circ\text{C}$.

Expression and purification of recombinant OtsB

Insect cells were grown in serum-free medium to a density of 2×10^6 cells/mL and a cell viability of greater than 95%. The cell culture (1 L) was transfected with the viral stock, 2.0×10^6 cells/mL. At 72 h post-infection, the cells were harvested by centrifugation. After freeze/thaw cycle, the pellet was resuspended in lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM MgCl_2 , 30 mM imidazole, 1 mM βME). DNase 3 mg, 1x Complete® protease inhibitor cocktail was also added in the pellet and lysed using a homogenizer. Cell debris was removed by centrifugation and the cell free extract passed through a 0.45 μm membrane filter. The filtrate was applied to a Ni-NTA column equilibrated in lysis buffer. After removal of the cell debris by centrifugation, the protein of interest was purified with a linear gradient of 10–300mM imidazole in 50 mM Tris-HCl, 200 mM NaCl, 5 mM MgCl_2 , 1 mM βME , pH 7.5, using Ni-NTA. The fractions with potential protein were concentrated using Vivaspin (5 MWCO) and then desalted on Hiload™ 26/60 Superdex™ 200 with 50 mM Tris-HCl, 5 mM

MgCl₂, 100 mM NaCl, pH 7.5. The sample was passed through an Acrodisc Mustang E membrane (Pall #MSTG25E3) to reduce endotoxin to 1.9 EU/mL. After the addition of 10% glycerol, the aliquots containing the recombinant enzyme were flash frozen, and stored at -80 °C. SDS-PAGE analysis revealed the presence of a protein with the correct molecular weight.

Small-scale synthesis of [¹⁹F]FDT using 3-Enzyme, One-pot System

To mimic 'hot' FDT synthesis, we tested 'cold' FDT synthesis from 0.138 mmol – 5.520 mmol scale. Reagent amounts are detailed in Supplementary Table S8. In summary, in a 1.5 mL Eppendorf tube were added the following: FDG (various concentrations, see Supplementary Table S8), ATP 0.72-7 mM, UDP-Glc 20 mM, KCl 100 mM, hexokinase 1 mg, OtsA 1 mg and OtsB 0.1 mg in 50 mM HEPES buffer solution containing 100 mM NaCl and 10 mM MgCl₂. The final reaction volume was adjusted to 1 mL in all cases. The reaction was incubated at 37 °C, 400 rpm and monitored by ¹⁹F-NMR and LC-MS. Full conversion was achieved in 45 min. A control sample of FDG with no enzyme added was also analyzed.

General Chemistry Experimental Procedures

Proton nuclear magnetic resonance spectra (¹H NMR) were recorded on a Bruker AVX 500 (500 MHz), a Bruker AVB 500 (500 MHz) or a Bruker AVB 400 (400 MHz) spectrometer as indicated. Carbon nuclear magnetic resonance (¹³C NMR) spectra were recorded on the same spectrometer as described above. ¹⁹F-NMR spectra were recorded on a Bruker AVX500, AVB500, AV600 and AVB400. NMR spectra were fully assigned using COSY and HSQC. All chemical shifts are quoted on the δ scale in ppm using residual solvents as the internal standards. Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet.

Infrared (IR) Spectra were recorded on a Bruker Tensor 27 Fourier Transform Spectrometer using KBr discs for solids and crystals. Absorption maxima (ν_{\max}) are reported in wave number (cm^{-1}) and classified as strong (s) or broad (br).

Low-resolution mass spectrometry (LRMS) were recorded using an agilent 6120 Quadrupole spectrometry using electrospray ionisation (ESI) in either positive or negative mode. High resolution mass spectra (HRMS) were recorded on a Thermo Orbitrap Exactive mass spectrometer. The instrument is calibrated with a standard calibration mix from thermo before the run. Liquid Chromatography – Mass spectrometry (LC-MS) analysis was carried out on a Waters Quattro Micro – MS with electrospray ionization operating in negative mode (ESI) interface with Agilent HPLC system and Waters 2777 auto sampler with 4-valve injector module. MS analysis was under the control of micro mass Masslynx 4.1 software.

Thin layer chromatography (TLC) was carried out using aluminium backed sheets coated with 60F254 silica gel (Merck). Visualization of the silica plates was achieved using a UV lamp ($\lambda_{\max} = 254 \text{ nm}$), and/or ammonium molybdate 5% in 2M H_2SO_4 . Column chromatography was carried out using Geduran® Si 60 silica gel (Merck). Mobile phases are reported in ratio of solvents (e.g. 4:1 petrol/ethyl acetate).

Radio-HPLC Method

Radio-HPLC analysis was carried out in a Bioscan radio-HPLC system (flow count) coupled with agilent 1200 HPLC system. Two analytical methods were used for the analysis of the depletion of starting material and formation of the product. In method 1, initially isocratic elution of 75:25 acetonitrile/water mobile phase combination was used with TSK gel normal phase HILIC column (phase amide -80, 10 cm x 4.6 mm x 5 μm) as stationary phase. The flow rate was maintained at 0.4 mL/min. This system resulted in the [^{18}F]FDG retention time of ~ 5.6 min and [^{18}F]FDT of 11.4 min. The mobile phase combination was then slightly

adjusted to 70:30 acetonitrile/water and hence retention time of [¹⁸F]FDG was 4.9 min and [¹⁸F]FDT was ~ 8.3 min. The total run time was 15 min.

The method 2 allowed the clear separation of phosphate sugars as well as analysis of [¹⁸F]FDG and [¹⁸F]FDT. In This method Advanced Glycan Mapping column (4.6 x 250 mm, 2.7 μm) was used as the stationary phase. The mobile phase was the gradient of two solvent system; solvent A= 50 mM ammonium formate, pH 4.5 and solvent B= acetonitrile. Flow rate was maintained at 0.5 mL/min and the total run time was 20 min.

Radio-TLC Method

Radio-TLC analysis was carried out in a Bioscan AR-2000 radio-TLC scanner. Silica gel plates 150 Å Silica Gel HL 250 cm 10 x 20 cm were used as the stationary phase and the 75:25 acetonitrile/water combination was used as the mobile phase. Once the strip developed to the 10 cm mark, was scanned by the radio-TLC scanner and analysed.

Luna-NH₂ Purification Method

[¹⁸F]FDT was purified by Sep-Pak cartridges. Sep-Pak Aminopropyl (NH₂) plus short cartridge (Waters, WAT020535, 360 mg) used as the stationary phase. The cartridge was pre-rinsed with 1 mL EtOH and then twice with 3 mL per wash sterile water for injection. A 10 mL syringe was attached to the cartridge with plunger removed to the 5 μm filter. 1 mL of EtOH was loaded on to the syringe.

The reaction mixture was diluted with 2 mL of EtOH. This mixture was poured into the above syringe with filter. Reaction vial was rinsed with further 1 mL of EtOH and transferred to the syringe. The contents of the syringe were filtered through 5 μm syringe tip filter to remove precipitate. Eluent was loaded onto the NH₂ cartridge and eluted without washing at a very low flow rate. Mixture was then concentrated first by evaporation and then by heating at

60 °C with a nitrogen flush. Finally the mixture was diluted with sterile water and filtered through sterile filter and drawn into the syringe.

OtsA Enzyme kinetic assay

The OtsA enzyme kinetic assay were measured through a NADH-linked spectrophotometric assay. The decrease in absorbance of NADH at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1}\text{cm}^{-1}$) was measured at 25 °C using a spectrophotometer equipped with a thermostat. To determine the kinetic parameters of UDP-Glc, various UDP-Glc concentrations (0.5 mM, 1 mM, 2 mM, 4 mM and 10 mM) were used at the fixed G6P concentration. The G6P concentrations were fixed at 2 mM, 4 mM, 8 mM, 16 mM and 35 mM. The reaction mixture contained 100 mM HEPES (pH 7.5), 10 mM MgCl_2 , 300 mM KCl, 1 mM potassium phosphoenolpyruvate, 300 μM NADH, 17 units of pyruvate kinase, and 24 units of lactate dehydrogenase in a total volume of 200 μL at 25 °C as well as a fixed G6P concentration and variable UDP-Glc concentration. For G6P assay, UDP-Glc concentration was fixed at 10 mM, whereas, G6P concentration was varied (1 mM, 2 mM, 4 mM, 8 mM, 16 mM and 32 mM). After incubation for 5 min at 25 °C, reactions were initiated by the addition of OtsA (final concentration 83 nM) then absorbance of the reaction mixture at 340 nm was recorded continuously.

TreT *P. horikoshii* Enzyme Kinetic Assay

TreT *P. horikoshii* enzyme kinetics were measured through a NADH-linked GT-continued spectrophotometric assay. The decrease in absorbance of NADH at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1}\text{cm}^{-1}$) was measured at 37 °C using a spectrophotometer equipped with a thermostat. To determine the kinetic parameters of UDP-Glc, various UDP-Glc concentrations (0.5 mM, 1 mM, 2mM, 4 mM and 10 mM) were used at a fixed Glc concentration of 50 mM. The reaction mixture contained 100 mM HEPES (pH 7.5), 10 mM MgCl_2 , 300 mM KCl, 1 mM

potassium phosphoenolpyruvate, 300 μ M NADH, 17 units of pyruvate kinase, and 24 units of lactate dehydrogenase in a total volume of 200 μ L at 37 °C as well as a fixed Glc concentration and variable UDP-Glc concentration. For Glc assay, UDP-Glc concentration was fixed at 20 mM, whereas, Glc concentration were varied (0.5 mM, 1 mM, 2 mM, 4 mM, 10 mM). After incubation for 5 min at 25 °C, reactions were initiated by the addition of TreT (final concentration 662 nM) then absorbance of the reaction mixture at 340 nm was recorded continuously.

TreT *T. tenax* Enzyme Kinetic Assay

In order to determine kinetic parameters (k_{cat} , K_m) of TreT *Thermoproteus tenax* enzyme, UDP-Glc concentrations were varied from 0.03125 mM – 7 mM. In 1.5 mL Eppendorf tube were added glucose 50 μ l (50 mM), MgCl₂ 20 μ l (10 mM), TreT enzyme 4 μ l, Hepes buffer varied volumes (50 mM, pH 7.5) to make up the total volume to 100 μ l. Different volumes of UDP-glucose were added to the reaction mixture so that the final concentration of UDP-glucose in the reaction mixture was 0.03125, 0.06125, 0.125, 0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00 and 7.00 mM. Reactions were started by adding 4 μ l enzyme and immediately placing on the thermoshaker at 70 °C. Each reaction was heated for 10 min and reactions were stopped by placing on ice and quenched by adding 20 μ l acetonitrile to precipitate the protein. Reactions were then flash-frozen with liquid nitrogen and stored at -20 °C. Prior to HPLC analysis each reaction mixture was centrifuged at 13000 rpm for 20 min. HPLC analysis was carried out by HPLC method 3 as specified in Supplementary Table S5. The kinetic parameters of TreT *T.tenax* were also determined based on variable acceptor substrate concentrations by linked assay as described for TreT *P. horikoshii* under the similar conditions. The amount of UDP-Glc was fixed at 20 mM and Glc concentration varied (0.5

mM, 1 mM, 2 mM, 4 mM and 10 mM). The reactions were incubated at 37 °C for 5 min and then enzyme (662 nM) added and reaction rates determined.

OtsAB Enzyme Kinetic Assay

OtsAB kinetic parameters were determined using HPLC method 3 as specified in Supplementary Table S5. We used variable UDP-Glc concentrations (2 mM, 4 mM, 8 mM, 16 mM and 32 mM) at a fixed G6P concentration of 80 mM. After adding the enzyme (1.23 μ M), reactions were incubated for 5 min at 37 °C in a thermoshaker. Reactions were then quenched by adding acetonitrile, centrifuged (13000 rpm, 10 min) and analysed by HPLC. A standard calibration curve of UDP was also generated under the same reaction and analysis conditions. From the resulting UDP calibration curve, unknown concentrations from enzymatic reactions were determined and plotted against the concentrations of G6P substrate to obtain the K_m and V_{max} values.

OtsB Enzyme Kinetics

For OtsB^{sf} enzyme kinetics we used ¹H NMR spectroscopy employing the ¹H anomeric resonances as primary signals that are well resolved and with low background. Initially, we analyzed standard samples of trehalose-6-phosphate (T6P) and trehalose. For kinetic assay, we incubated several concentrations of T6P (0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM, 4.0 mM and 5.0 mM). Reaction was initiated by adding 10 μ L of OtsB and incubated at 37 °C for 10 min. After 10 min, reactions were stopped by boiling at 95 °C for 5 min and then analysed by ¹H NMR using D₂O as a solvent. A standard calibration curve for trehalose was also generated and, using this calibration curve, concentrations of resulting trehalose from enzymatic reaction were obtained and analyzed through non-linear regression in GraphPad prism to give a Michaelis-Menten curve.

The activity of OtsB *E. coli* was also tested using commercially available Abcam Phosphate assay kit. By determining the amount of inorganic phosphate released from T6P by action of OtsB, enzymatic activity was to be calculated. First, 10 μL of 10 mM phosphate standard was added to 900 μL of buffer (50 mM Tris, 5 mM MgCl_2 , pH 7.5) to generate 100 μM standard phosphate solution. 0, 10, 20, 30, 40 and 50 μL of 100 μM standard phosphate solution were added to individual wells of a 96-well plate. The volume was adjusted to 200 μL with dH_2O to generate phosphate standards. 30 μL of working reagent was added to all wells and was mixed well. The plate was incubated at 37 $^\circ\text{C}$ for 30 minutes. Absorbance was read at 650 nm. A calibration curve was thus generated. As a practical note, in earlier experiments, reaction aliquots were mixed with 30 μL to quench the reaction and then volume was made up to 200 μL ; it was found that doing so leads to precipitation. Therefore, in later experiments, reaction aliquots were diluted in the well using the reaction buffer, followed by addition of working reagent. In this way, no precipitate was observed. Next, enzymatic reactions were conducted in a final volume of 200 μL containing the following components: 5 mM MgCl_2 , 50 mM Tris buffer, pH 7.5, OtsB and T6P. 1.720 μM of enzyme was used in each case. T6P concentration was varied from 0.5 mM to 3 mM with an increment of 0.5 mM. Reaction aliquots were taken from the reaction at time, $t = 0.5$ min, 1 min, 2 min, 2.5 min, 3 min, 3.5 min, 4 min, 5 min, 6 min, 8 min and 10 minutes. The volume of reaction aliquots was taken in such a way that when diluted with reaction buffer, the maximum possible amount of phosphate remained within the linear range of the assay. 30 μL of working reagent was added after dilution. Samples were incubated at 37 $^\circ\text{C}$ for 30 min. Absorbance was read at 650 nm. Using the calibration curve, the rate of formation of P_i was calculated and plotted against time to give rate of reaction at a particular substrate concentration. Data for different substrate concentrations were analyzed using non-linear regression in GraphPad prism to give a Michaelis-Menten curve.

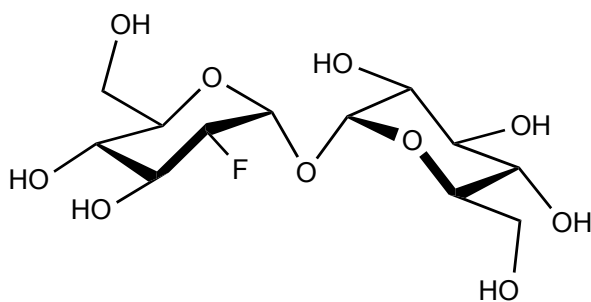
Chemoenzymatic Reaction Optimisation and Scale-up

During reaction optimisation we tested the effect of ATP concentrations to the conversion of FDG to FDG6P initially at 1 mg scale. We set-up four different reactions in 1.5 mL Eppendorf tubes as shown in Supplementary Table S7. Reactions were incubated at 37 °C for 1 h at 400 rpm in a thermoshaker and then analysed by ¹⁹F-NMR spectroscopy to observe the conversion.

To test the OtsA and OtsB enzymes needed for a reaction, we tested several concentrations of OtsA (6.65 μM, 13.3 μM, 18.2 μM and 36.6 μM) and OtsB (1.53 μM, 3.06 μM, 6.13 μM, 12.26 μM) in a 50 mg scale while maintaining all the other reaction parameters constant. Reactions were incubated at 37 °C at 400 rpm for 1 h and then analysed by ¹⁹F-NMR spectroscopy.

In order to further optimise the enzymatic reaction conditions using 3 enzyme synthesis method, we tested various substrate concentrations i.e. with fixed enzyme concentrations. In summary in a 50 mL falcon tube were added FDG (various FDG concentrations i.e. 6.25 mg, 12.5 mg, 25 mg and 50 mg), ATP with equivalent or slight excess molar concentration to FDG, hexokinase 5 mg (680 U), UDP-Glc (37 mg with final concentration of 30 mM), OtsA enzyme (2 mg, 18 μM), OtsB (0.4 mg, 6.2 μM) and KCl (100 mM). Reaction volume was made up to 2 mL by HEPES buffer solution (HEPES 50 mM, pH 7.5, 100 mM NaCl, MgCl₂ 10 mM). Reactions were incubated at 37 °C and samples were taken after 15 min, 30 min, 60 min, 120 min, 200 min and 690 min, diluted with D₂O and analysed by ¹⁹F-NMR spectroscopy. 50 mg reaction was further optimised with additional UDP-Glc (168 mg, 138 mM) and analysed by ¹⁹F-NMR.

Chemoenzymatic Synthesis of FDT



In a 50 mL sterile falcon tube were added; FDG (100 mg, 367 mM), ATP (320 mg, 368 mM) and $MgCl_2$ (5 mg). The contents were dissolved in HEPES buffer solution containing 100 mM NaCl (100 mM, 1 mL, pH 7.5). The pH was then adjusted to 8.0 with NaOH. Hexokinase (0.6 mL) was added to the reaction mixture to initiate the reaction. The reaction mixture was incubated at 30 °C, 50 rpm for 1-2 hours. Phosphorylation of FDG to FDG6P was monitored by TLC and ^{19}F -NMR spectroscopy. Full conversion to FDG6P was observed within 2 hours for each batch synthesized.

Within the same pot, UDP-Glc (200 mg, 110 mM), KCl (100 mM) and OtsA enzyme (26 μ M) was added. The total reaction was then adjusted to 3 mL. The reaction mixture was incubated to similar conditions as above for 24 h to allow appropriate conversion to FDT6P. The conversion of FDG6P to FDT6P was monitored by ^{19}F -NMR spectroscopy. The average maximum conversion after 24 h was between 65-70 %. After 24 h, in the same reaction mixture OtsB enzyme (6.23 μ M) was added. The pH was adjusted to 8.0 and reaction mixture was incubated at 37 °C, 50 rpm. Reaction progress was monitored by TLC and ^{19}F -NMR. The reaction was usually complete within 2 hours.

Purification and Characterisation of FDT

The enzymes from crude reaction mixture were precipitated by either heating the mixture at 95 °C for 10 min or adding 2 mL EtOH and isolated by centrifugation (Vivaspin, 10,000 MWCO) and/or filtration. The resulting filtrate was lyophilized and the crude mixture was

further precipitated with MeOH/MeCN 90:10 solution and then purified by flash column chromatography. For purification normal phase silica was used as stationary phase and mixture of Ethyl acetate: Iso-propanol and water (48%/48%/4%) were used as mobile phase. FDT was separated from residual FDG6P and residual FDG. The fractions containing FDT were pooled, concentrated and then lyophilised to dryness. The Lyophilisation step was repeated for a further 2-3 times to get rid of any residual water. The freeze dried compound was then characterised by proton (^1H NMR), carbon (^{13}C NMR), fluorine (^{19}F NMR) and ESI-MS. $[\alpha]_{\text{D}}^{25} + 128.9$ (c 1.00, H_2O). ^1H NMR (500 MHz, D_2O) δ 5.33 (d, $J = 3.9$ Hz, 1H, H-1), 5.11 (d, $J = 3.7$ Hz, 1H, H-1'), 4.40 (ddd, $J = 49.0, 9.6, 3.9$ Hz, 1H, H-2), 4.02 (dt, $J = 13.2, 9.4$ Hz, 1H, H-3), 3.79 – 3.62 (m, 7H, H-5, H-6a, H-6b, H-3', H-5', H-6a', H-6b'), 3.56 (dd, $J = 9.9, 3.7$ Hz, 1H, H-2'), 3.41(t, $J = 9.7$ Hz, 1H, H-4) , 3.35 (t, $J = 9.4$ Hz, 1H, H-4') ppm. ^{13}C NMR (125 MHz, D_2O) δ 94.0 (C-1'), 91.2 (d, $J_{\text{F,C-1}} = 21.8$ Hz, C-1), 89.5 (d, $J_{\text{F,C-2}} = 186.6$ Hz, C-2), 72.6 (C-3'), 72.2 (C-5 and C-5'), 71.4 (d, $J_{\text{F,C-3}} = 15.8\text{Hz}$, C-3) 70.9 (C-2'), 69.6(C-4'), 69.1 (d, $J = 8.0$ Hz, C-4), 60.5 (C-6 or C-6'), 60.3 (C-6 or C-6') ppm. ^{19}F NMR (470 MHz, D_2O) δ – 201.18 ppm. IR (neat) ν 3264 (OH), 1647, 1409, 1150, 1103, 990, 948, 841, 802, 705 cm^{-1} . ESI-MS: ESI-MS calculated for $\text{C}_{12}\text{H}_{21}\text{F}_1\text{O}_{10}\text{Na}_1$ $[\text{M} + \text{Na}]$ 367.1011, found 367.1011.

Stability Testing of FDT Solution in HEPES Buffer

In order to establish the storage conditions of the final formulation of an injection, small scale stability testing of the final formulation was carried out. In summary, FDT sample was dissolved in HEPES Buffer solution (50 mM, pH 7.5) containing 7.5 mM MgCl_2 . One portion of the dissolved solution was stored at room temperature (20 – 25 $^\circ\text{C}$) and the other portion was stored between 2-8 $^\circ\text{C}$. Samples were taken at day 0, 3, 5, 7 and 10 and analysed by ^{19}F

NMR spectroscopy in triplicate. The means of the obtained integrals are then plotted against the day of testing. In addition, spectras were observed for any additional peaks as well.

Stability Testing of FDT Solid

In order to ensure that the compound will be stable throughout the testing period, on-going stability testing of the FDT sample from solid was also investigated over nine months' period. Material for testing was stored at - 20 °C and periodic testing was carried out by dissolving ~ 8 mg of sample in D₂O and then subsequently analysed by ¹H NMR spectroscopy.

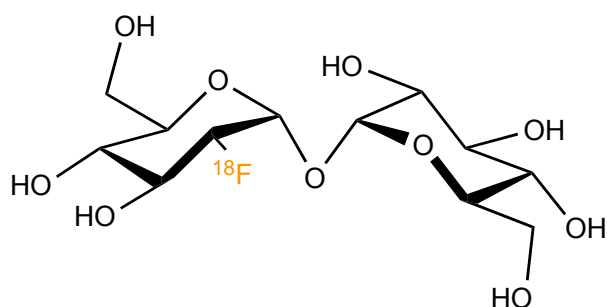
Determination of Purity by ¹H NMR

Purity of the final compound was determined by ¹H NMR spectroscopy by determining relative concentration of the substance as compared to impurities. Known quantity of FDT was dissolved in D₂O and quantified by ¹H NMR spectroscopy. Maleic acid was used as an internal standard.

²³Na-NMR Quantification

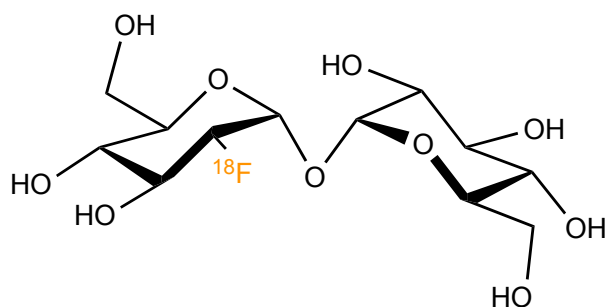
In order to determine the amount of salt (NaCl) in the FDT compound we used ²³Na-NMR in AVX500 instrument. We generated the calibration curve of NaCl solution of known concentration and then run the FDT samples using the same parameters as NaCl standards. The amount of Na was calculated from the standard calibration curve as an unknown and then from molar ratio of Na:FDT the amount of salt then determined.

[¹⁸F]FDT Synthesis using TreT *P. horikoshii*



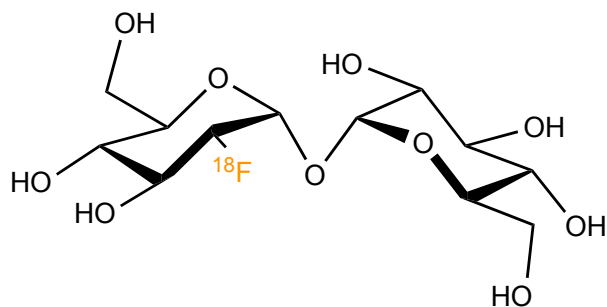
In a 2 mL Eppendorf tube were added UDP-Glc (30 μ L of a 1M aq solution), TreT enzyme (0.7 mg). Reaction was started by adding [¹⁸F]FDG (500 μ L, 14.1 mCi/mL). Reaction volume was made up to 1 mL by adding HEPES buffer solution (100 mM HEPES, 10 mM MgCl₂, pH 7.5). The reaction was incubated at 37 °C in a thermoshaker and monitored by radio-HPLC. Once [¹⁸F]FDG fully converted to [¹⁸F]FDT, the reaction mixture was purified on a Luna-NH₂ matrix as described in the Methods section.

[¹⁸F]FDT Synthesis using 3 Enzymes in one pot



In a 2 mL Eppendorf tube were added UDP-Glc (30 μ L of a 1M aq solution), ATP (10 mM), hexokinase (5 mg, 640 U), OtsA (0.7 mg, 12.3 μ M), OtsB (0.4 mg, 12.5 μ M). [¹⁸F]FDG (500 μ L, 15.6 mCi) was added to the reaction mixture to start the reaction. Final volume of reaction was adjusted to 1 mL by adding HEPES buffer solution and incubated at 37 °C in a thermoshaker. Reaction progress was monitored by radio-HPLC and stopped after 60 min when fully converted to the product and purified by Luna-NH₂ cartridge method.

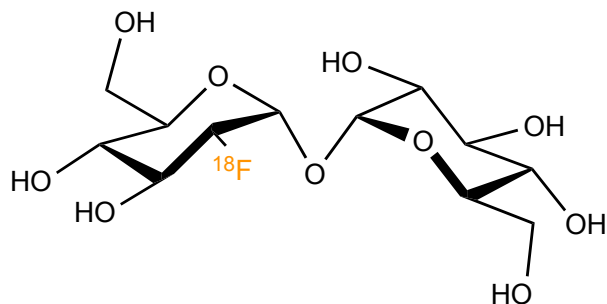
[¹⁸F]FDT Synthesis using 2 Enzymes in one pot



In a 2 mL Eppendorf tube were added UDP-Glc (30 μ L of a 1M aq solution), ATP (10 mM), OtsAB fusion enzyme (0.1 mg, 1.3 μ M), hexokinase (2 mg, 256 U). The reaction was started by the addition of [¹⁸F]FDG (250 μ L, 3.5 mCi). The reaction volume was adjusted to 1 mL by adding HEPES buffer solution (HEPES 100 mM, MgCl₂ 10 mM, NaCl 100 mM, pH 7.5). The reaction was started by incubating at 37 °C and monitored by radio-HPLC. The reaction did not work under the conditions described above even after 90 min of incubation.

In an another attempt, higher enzyme concentrations were added into the reaction mixture and incubated for 2 h at 37 °C but there was no conversion at all.

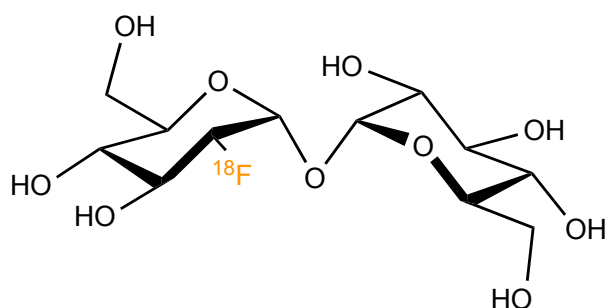
Automation of [¹⁸F]FDT Synthesis



[¹⁸F]FDG (1480- 3700 MBq; 40-100 mCi in 0.8 -1.8 mL) was transferred under vacuum to the Reactor 1 containing 100 μ L 1 M HEPES buffer, pH 7.6, 20 μ L 1 M MgCl₂, 20 μ L 1 M

ATP, 60 μ L 1 M UDP-Glc, \sim 50 μ L OtsA (1 mg), \sim 50 μ L OtsB (1 mg), 20 μ L hexokinase (5 mg). The reaction mixture was incubated for 30 min at 45 $^{\circ}$ C and ethanol was added (3 - 6 mL). The reaction mixture was passed through the filter (5 μ m) and a stack of three NH₂-cartridges and collected in Reactor 2. An additional 1 mL 75% ethanol in water was added to rinse the Reactor 1 and transferred into the Reactor 2. The combined solution was concentrated under nitrogen at 60 $^{\circ}$ C for 10 min. 2 mL saline was added to the Reactor 2. The final [¹⁸F] FDT solution was transferred to the product vial through a sterile filter (0.22 μ m). The quality of the product was determined by analytical radio-HPLC (Condition: 4.6 x 250 mm, 2.7 μ m AdvanceBio Glycan Mapping Column; solvent A = 50 mM ammonium formate, pH 4.5, solvent B = acetonitrile; Flow rate = 0.5 mL/min; gradient 0-15 min 68-62% B; 15-20 min 62-68% B). Identity of the compound was confirmed by LC-MS.

Chemoenzymatic synthesis of [¹⁸F]FDT for Animal Studies



For the animal studies, [¹⁸F]FDG (20-30 mCi in 0.8 -1 mL) was injected to the reaction mixture containing 100 μ L 1 M HEPES buffer, pH 7.6, 20 μ L 1 M MgCl₂, 20 μ L 1 M ATP, 60 μ L 1 M UDP-Glc, \sim 50 μ L OtsA (1 mg), \sim 50 μ L OtsB (1 mg), 20 μ L hexokinase (5 mg). The reaction mixture was incubated at 37 $^{\circ}$ C for 30 min. After 30 min, the mixture was diluted with absolute ethanol (4 mL) and passed through a 5 μ m syringe filter. The eluent was passed slowly through an amine Sep-Pak SPE cartridge at a flow rate of 1-2 drops per second.

The eluent was then concentrated in vacuo. The resulting solution was filter-sterilized into a sterile vial for delivery. Identity of the compound was confirmed by LC-MS.

Specific Activity Analysis of [¹⁸F]FDT

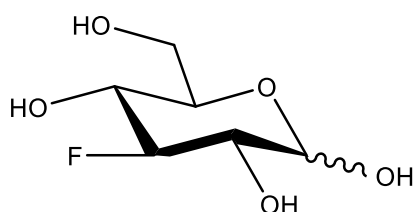
The radioactivity of the final products, [¹⁸F]FDT, was obtained using a radiation detector. To quantify the mass of the decayed [¹⁸F]FDT in the form of [¹⁸O] trehalose, a modified version of the trehalose quantification enzymatic assay was performed.¹ A trehalose standard calibration curve (linear fit, $R^2 = 0.9984$) was generated using known concentrations of trehalose. The ‘cold equivalent’ masses of [¹⁸F]FDT from syntheses were calculated based on this calibration curve. Then, the specific activity of the final product was calculated, following the definition, radioactivity at the end of the synthesis / unit mass of compound.

Western Blot Analyses of [¹⁸F]FDT Samples

Samples were run on a 4–12% Bis-Tris NuPAGE gel (ThermoFisher) using MOPS buffer and transferred to a PVDF membrane using an iBlot 2 transfer system at 25 V for 7 min. Membranes were blocked with a 5% milk solution (50 mM Tris HCl, pH6; 150 mM NaCl; 0.1% Tween-20) and primary antibodies were incubated overnight at 4 °C in blocking solution. Primary antibodies were Mouse Anti-6XHis (Invitrogen # MA1-135) for OtsA and OtsB detection and Rabbit Anti-Hexokinase (Bio-Rad #4959-9988, 1:3000) for yeast hexokinase detection. After rinsing with TBST, membranes were incubated at 4 °C with rocking for 1h with Goat anti-Mouse IgG-AP Conjugate (Bio-Rad #1706520, 1:1000) for OtsA and OtsB, and Sheep Anti-Rabbit IgG:DyLight®488 (Bio-rad # STAR36D488GA, 1:5000) for yeast hexokinase. The membrane probed with anti-Mouse IgG-AP Conjugate was

further incubated with BCIP/NBT alkaline phosphate substrate (Bio-Rad #1706432) after TBS-T washes. A chemiluminescent signal was captured using a Bio-Rad Chemidoc system.

Chemical synthesis of [¹⁹F]-3-FDG



In a 100 mL flask was dissolved 0.50 g of diacetone allofuranose (1.92 mmol, 1 eq) and 0.50g of *N,N*-diaminopyridine (4.1 mmol, 2.1 eq) in 20 mL of dry dichloromethane. The mixture was cooled down to -20°C using an acetone/dry ice bath then 0.5 mL of diethylaminosulfur trifluoride

(DAST, 3.8 mmol, 2.0 eq) was added slowly, then the solution was allowed to return slowly to room temperature while stirring overnight. TLC (95:5 CH₂Cl₂:MeOH) showed a single compound (R_f 0.77). The mixture was cooled to 0 °C and 5mL of methanol was added slowly. The mixture was stirred 1h and evaporated to dryness to give a yellow oil that was purified by silica column chromatography (49:1 CH₂Cl₂:EtOAc then 24:1 CH₂Cl₂:EtOAc) to give 416 mg of a colourless oil, that was used in the next step.

In a 50mL flask was dissolved 416mg of diacetone 3-FDG (1.58 mmol) in 20mL of water and 4 mL of ethanol. Ca. 6 mL of activated DOWEX 50W-H8 was added and the mixture was stirred for 92 hours. The mixture was filtered, and the liquid was evaporated and dried under high vacuum to give 240.9 mg of crude compound. This crude compound was dissolved in a mixture 7:2:1 ethyl acetate:ethanol:water and filtered through a short plug of silica, then the silica was washed with 200mL of the same mixture. The solution was evaporated and dried under high vacuum to give 128 mg of a colorless oil in 44% overall yield.

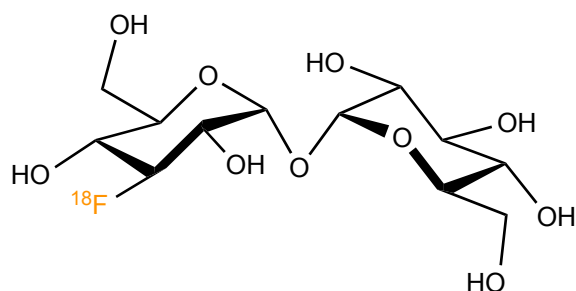
Analysis: ^1H NMR (400 MHz, D_2O): 5.14 (1H, app t, 4.0 Hz, H-1 α), 4.56 (1H, d, 8.0 Hz, H-1 β), 4.49 (1H, dt, $J_{\text{H-F}}$ 52 Hz, 8 Hz H-3) 4.31(1H, dt, $J_{\text{H-F}}$ 52 Hz, 8 Hz, H-3), 3.80-3.40 (10H, m, H-2, H-4, H-5,H-6, H-6'); ^{13}C NMR (100.6 MHz, D_2O): 96.8 (d, $J_{\text{C-F}}$ 181 Hz C-3), 95.3 (d, $J_{\text{C-F}}$ 179 Hz C-3'), 95.5(d, $J_{\text{C-F}}$ 12 Hz C-1 beta), 92.5 (d, $J_{\text{C-F}}$ 10 Hz C-1 α), 74.9 (d, $J_{\text{C-F}}$ 9 Hz), 72.9 (d, $J_{\text{C-F}}$ 16 Hz), 71.1(d, $J_{\text{C-F}}$ 7 Hz), 70.3 (d, $J_{\text{C-F}}$ 17 Hz), 68.3 (C-5), 68.1 (C-5), 60.6(C-6), 60.5(C-6); ^{19}F NMR(376.5 MHz, D_2O): β -195.2, dt, $J_{\text{H-3-F}}$ 52.7 Hz, $J_{\text{H-4-F}} = J_{\text{H-2-F}} = 15.0$ Hz; α -200.1, dtd, $J_{\text{H-3-F}}$ 52.7 Hz, $J_{\text{H-4-F}}$, $J_{\text{H-2-F}}$ 15.0 Hz, 4.0 Hz; MS (ESI $^+$) [$\text{M}+\text{Cl}$] $^-$ found 217.05 and 219.05, require 217.0279 and 219.0250.

1,2:5,6-di-*O*-isopropylidene-3-*O*-triflyl- α -D-allofuranose

260 mg of 1,2:5,6-di-*O*-isopropylidene D-allofuranose (1 mmol, 1eq) and 308 mg of 2,6-di-tertbutyl-4-methylpyridine (1.5 mmol, 1.5 eq) were dissolved in 10 mL of dichloromethane. 220 μL of trifluoromethanesulfonic anhydride (1.3 mmol, 1.3 eq) were added slowly at room temperature and the mixture was stirred overnight at room temperature. TLC showed completion of the reaction, and the mixture was evaporated to dryness under vacuum at less than $+30^\circ\text{C}$. The residue was purified by silica column chromatography (95:5 CH_2Cl_2 :EtOAc) to give 309.7 mg of as a colorless oil (0.788 mmol, 79%).

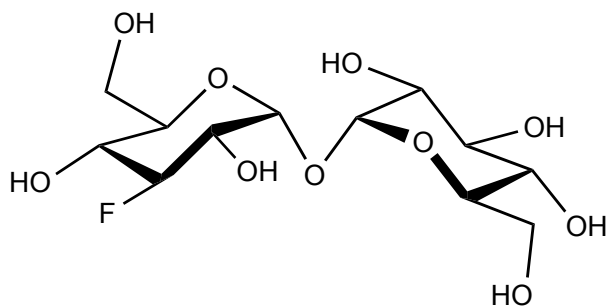
Analysis: TLC $R_f=0.85$ (80:20 CH_2Cl_2 :EtOAc); MS (ESI $^+$) found 415.05, [$\text{M}+\text{Na}^+$] requires 415.0645; ^1H NMR (400 MHz CDCl_3) δ ppm 5.84 (1H, d, $J_{1,2}=4.0\text{Hz}$, H-1), 4.91 (1H, app t, 6.2 Hz, H-3), 4.78 (1H, app t, 4.0 Hz), 4.19 (2H, m, H-4 and H-5), 4.12 (1H, dd, $J_{6a-6b}=8.8\text{Hz}$, $J_{6a-5}=6.2$ Hz, H-6a), 3.92 (1H, dd, $J_{6b,6a}=8.8\text{Hz}$, $J_{6b,5}=4.8$ Hz, H-6b), 1.59, 1.45, 1.39, 1.35 (12H, 4s, 4 x CH_3 isopropylidene). ^{13}C NMR (101 MHz CDCl_3) δ ppm 114.4, 110.3 (isopropylidene acetal), 104.1 (C-1), 82.9 (C-3), 77.8, 77.7 (C-2 and C-4 or C-5), 75.1 (C-5 or C- 4), 66.3 (C-6), 26.9, 26.5, 26.2, 24.8 (4 x CH_3 isopropylidene), CF_3 OTf weak. ^{19}F NMR (376.5 MHz, CDCl_3) δ ppm -74.9, s, CF_3 OTf.

Attempted Chemoenzymatic Synthesis of [¹⁸F]-3-FDT



To a 100 μ L solution of scintomics dried K[¹⁸F-]/K222 solution (activity 193.2 MBq at 12:40, was added 200 μ L solution of 3-OTf diisopropylidene allofuranose (18.4 mg in acetonitrile). The mixture was heated at 110 °C for 10 min, after which it was cooled down to room temperature. 200 μ L of 5 N HCl solution was added into the reaction mixture and the solution was heated at 110 °C for further 10 min, cooled to room temperature and monitored by TLC for the formation of [¹⁸F]-3-FDG. 92 MBq of [¹⁸F]-3-FDG in 0.5 M phosphate buffer solution (phosphate buffer pH 7.5) from the above reaction mixture was used in an attempted enzymatic one pot synthesis. Following reagents were added : 12 μ L OF 1M ATP, 45 μ L of 1 M UDP-Glc, 12 μ L 1M MgCl₂, 100 μ L OtsA (23 mg/mL) and 35 μ L OtsB. The reaction vial was incubated at 30 °C and monitored by radio-TLC over 4h.

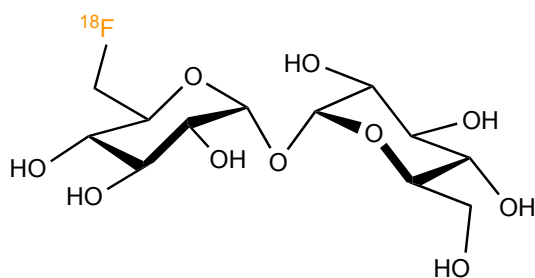
Chemoenzymatic Synthesis of [¹⁹F]-3-FDT



A one-pot reaction mixture containing 2.5 mM 3FGlc, 8.3 mM ATP, 33 mM UDP-glucose, 1.3 mg/mL OtsA and 3.4 mg/mL OtsB was prepared in pH 7.25, 50 mM Hepes buffer.

Hexokinase (5.0 mg/mL final concentration) was added to initiate the reaction. Reaction mixture was incubated at 30 °C and monitored by ESI-MS at every 10 minute interval. Alternatively, aliquots were withdrawn at every 30 minutes. Withdrawn aliquots were frozen on liquid nitrogen and boiled for 1 minute. Proteins were filtered off using a Vivaspin (Sartorius, 10K NMWCO) and each aliquot was analyzed by HPLC (Dionex UltiMate 3000) with the column, Phenomenex Luna NH₂ (4.6 × 250 μm). The column was eluted with isocratic 7/3 acetonitrile/water. The maximum concentration of OtsB that could be used was 3.4 mg/mL. Above this concentration, OtsB precipitated while the reaction did not proceed to a meaningful extent at lower than this concentration of OtsB. By ESI/MS, it was found that the reaction did not go completion in 2 hours, after which precipitation formed. Based on peak intensity it was estimated that a mixture 7:1 3FTre:3FGlc was formed. HPLC analysis also revealed poor separation.

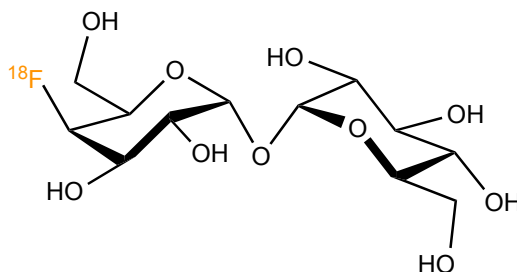
Radiochemical synthesis of [¹⁸F]-6-FDT



To a 13 x 100 TT, were added 100 μL K222 solution and 60 μL K₂CO₃, and 20 μL ¹⁸F- (3.20 mCi). The reaction contents were evaporated under stream of argon in a 105 °C heat block. The reaction mixture was then azeotroped 3 times with 200 μL of CH₃CN. Triflate precursor, trehalose heptaacetate triflate was dissolved in 300 μL of CH₃CN and added into the reaction mixture and incubated at +55 °C for 15 min. After 15 min, 30 μL of 5 M NaOMe (150 μmol) was added and the reaction was allowed to stand for 5 min. After 37 min, reaction was

monitored by analytical HPLC (Luna NH₂ 4.6 x 250 mm; isocratic elution of 80% CH₃CN and 20% H₂O; flow rate 1 mL/min). 6-[¹⁸F]-Tre eluted at 8.5 min. The reaction contents were then purified by preparative HPLC (Luna NH₂ 10 x 150 mm; isocratic elution of 80% CH₃CN and 20% H₂O; flow rate 4 mL/min). The desired compound was obtained in 32% yield (\pm 4%, non-decay corrected, n=2, from preparative HPLC) over 50-55 minutes total reaction time.

Radiochemical synthesis of 4-epi-[¹⁸F]-FDT



To a 13 x 100 TT, was combined aqueous potassium carbonate (0.1 M, 60 mL, 12 mmol, 2.4 eq), K222 (4.5 mg, 12 mmol, 2.4 eq) as a solution in MeCN (100 mL, 120 mM), and [¹⁸F]-fluoride (120 mL, 100 mCi). The mixture was azeotroped at 110 °C, washing with MeCN (3 x 200 mL). To this dried mixture was added substrate (5 mg, 6.5 mmol, 1 eq) in MeCN (200 mL, 25 mM), and the reaction was heated at 120 °C for 15 min. the resulting brown solution was diluted with saline (154 mM NaCl, 15 mL) and passed slowly through a C₁₈ Sep-Pak. The Sep-Pak was then washed with water (1 x 10 mL), then treated with NaOH (1 M, 200 mL). The NaOH was dispensed onto the Sep-Pak, which was allowed to stand for 5 min at room temperature. The deprotected product was then eluted with water (3 mL), acidified with HCl (1 M, 150 mL) and buffered with saline-sodium citrate (10X, 300 mL). The neutralized material was purified by passage through an Alumina Sep-Pak Plus, followed by a C₁₈ SPE). Material was analyzed for radiochemical purity by HPLC and TLC. HPLC was performed on an Agilent 1100 on a Phenomenex Luna -NH₂ 4.6 x 250 mm column using an

isocratic flow rate of 80:20 MeCN:H₂O at 1 mL/min. TLC analysis was performed using multichannel silica plates with a cellulose preabsorbing zone developed in a mixture of 1:4:4 water:isopropanol:ethyl acetate.

Syntheses were performed in a 21-150 mCi scale yielding 9-66 mCi of epi-4-F-Tre, with an average radiochemical yield (n=10) of 33.7 (+/- 8%, non-decay corrected) and an average synthesis time of 57 min.

Supplementary Figures

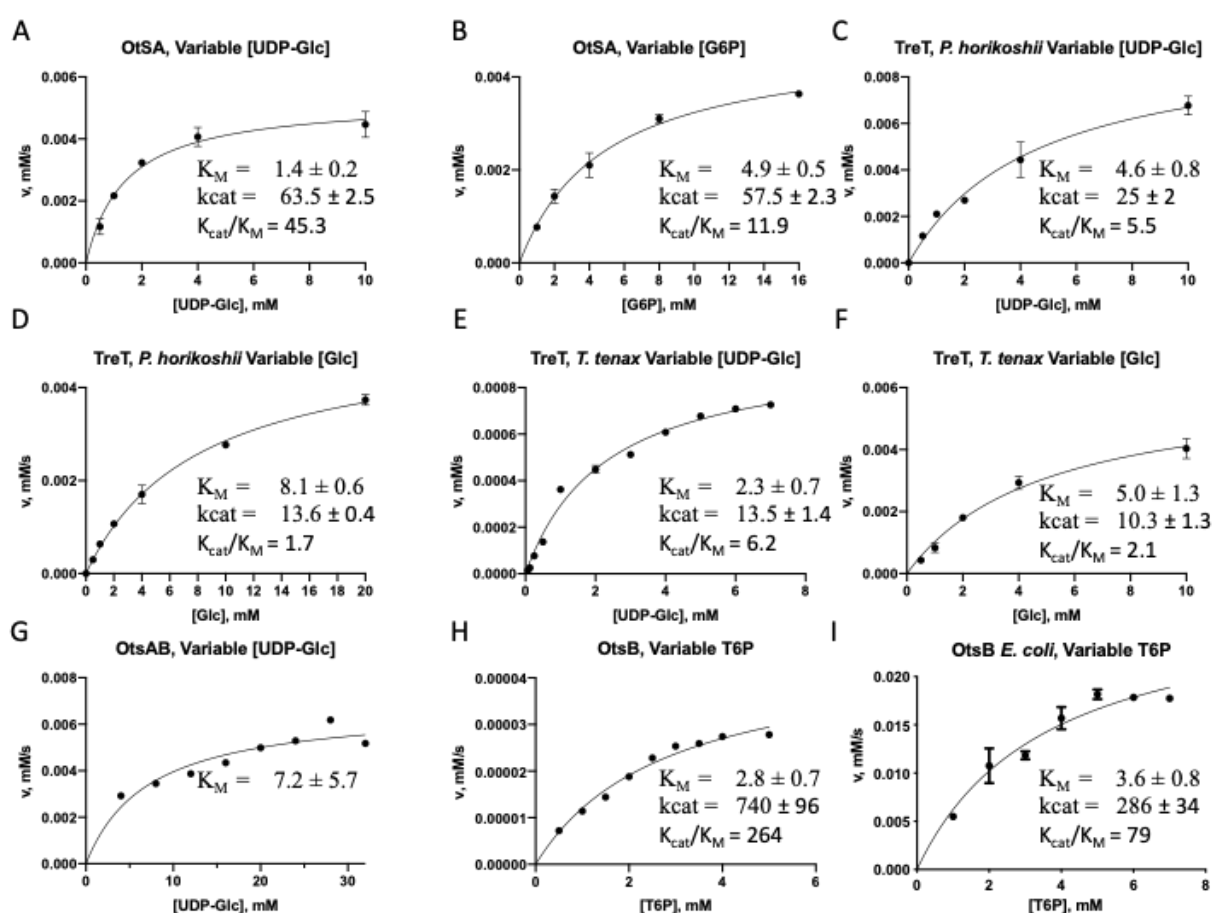


Figure S1. Kinetic parameters donor and acceptor substrates of OtsA, TreT, OtsAB fusion enzyme and OtsB enzyme.

A) Michaelis-Menten plot showing the activity of OtsA as a function of UDP-Glc with a fixed Glc-6P concentration (n = 3). **B)** Michaelis-Menten plot showing the activity of OtsA as a function of Glc-6P with a fixed UDPG concentrations (n = 3). **C)** Michaelis-Menten plot showing the activity of TreT *P. horikoshii* as a function of UDP-Glc with a fixed Glc

concentration (n = 3). **D)** Michaelis-Menten plot showing the activity of TreT *P. horikoshii* as a function of Glc with a fixed UDP-Glc concentrations (n = 3). **E)** Michaelis-Menten plot showing the activity of TreT *T. tenax* as a function of UDP-Glc with a fixed Glc concentration (n = 3). **F).** Michaelis-Menten plot showing the activity of TreT *T. tenax* as a function of Glc with a fixed UDP-Glc concentration (n = 3). **G).** Michaelis-Menten plot showing the activity of OtsAB fusion as a function of UDP-Glc with a fixed G6P concentration (n = 1). **H)** Michaelis-Menten plot showing the activity of OtsB^{sf} as a function of T6P (n = 2). **I)** Michaelis-Menten plot showing the activity of OtsB as a function of T6P. Units for kinetic parameters are s⁻¹ for k_{cat} and mM for K_M (n = 2). n values are for technical replicates of representative samples. The data points are average values from given values of n with error bars \pm SD.

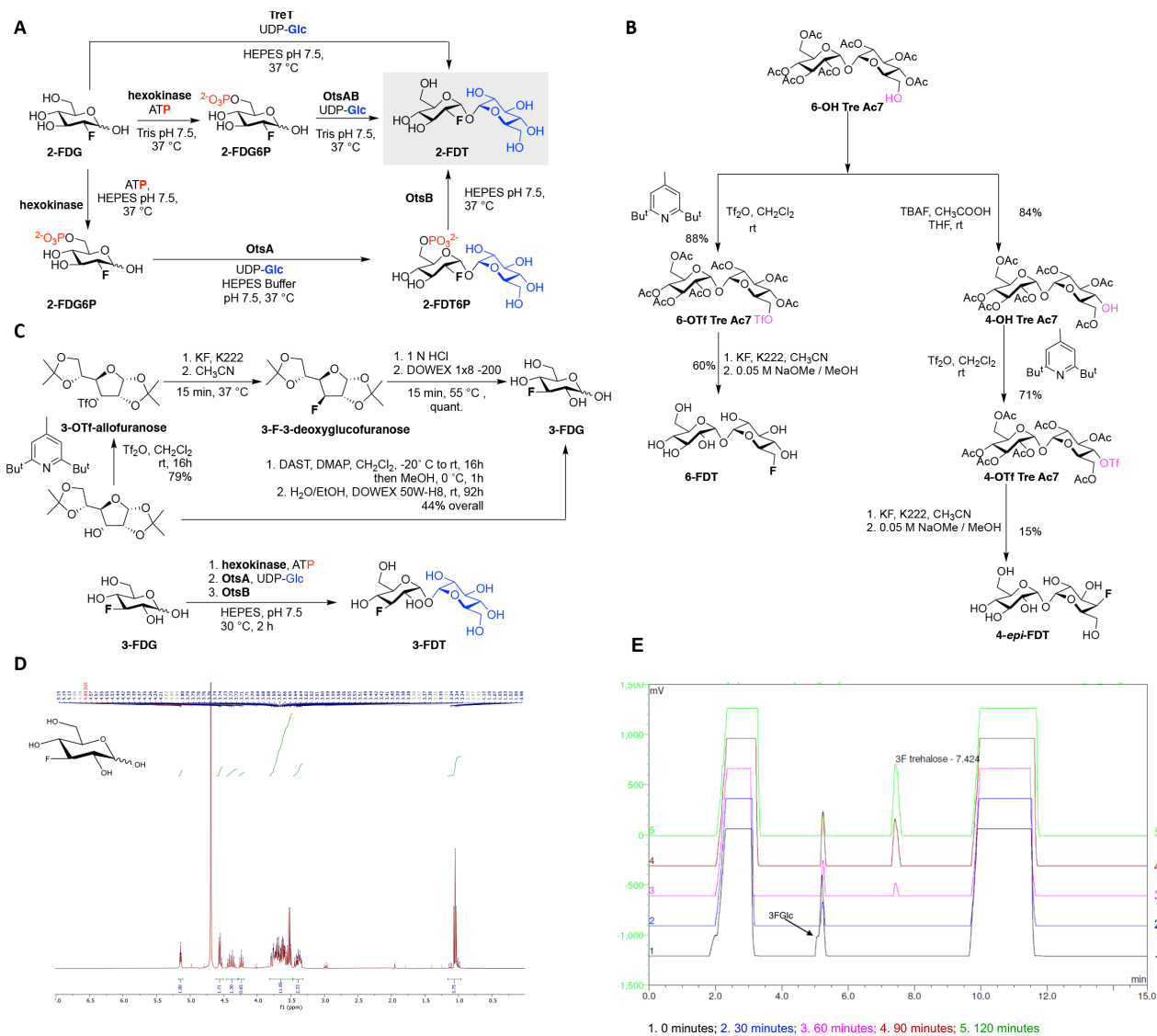
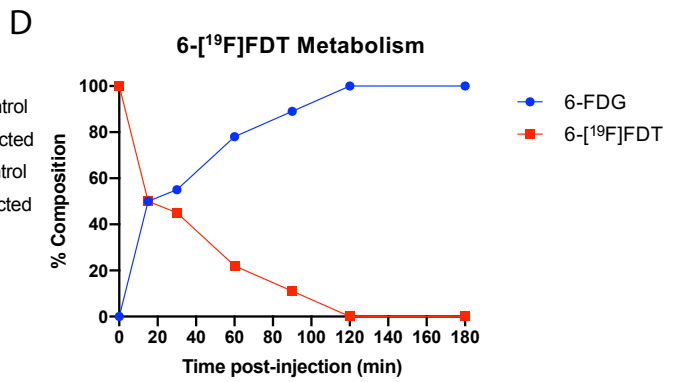
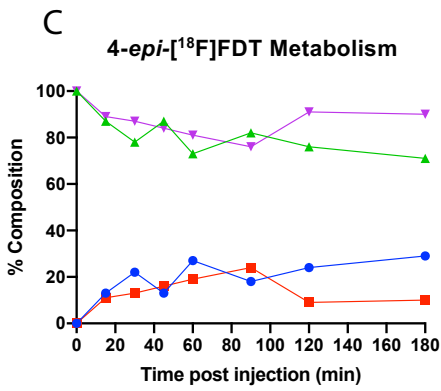
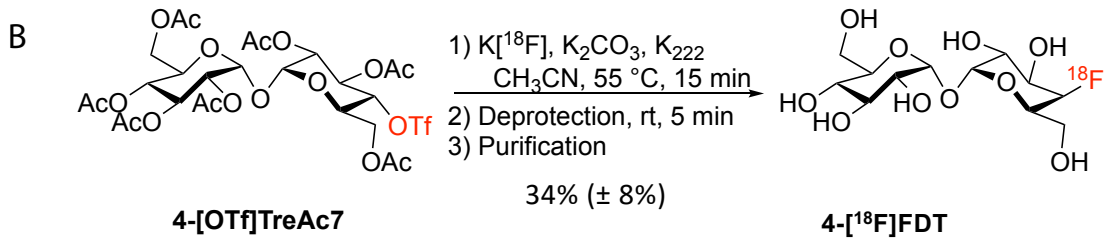
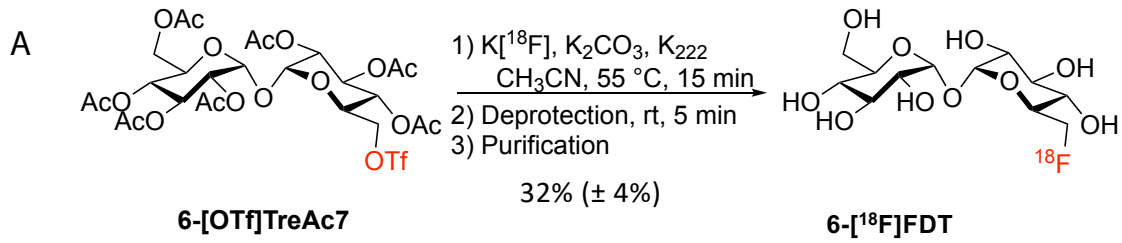


Figure S2. Exploration of ‘Cold’ Synthetic Routes to FDT variants.

The ‘cold’ synthesis of 4 different ¹⁹F-containing FDT variants (termed here 2-FDT – described also as FDT in the main text –, 3-FDT, 4-*epi*-FDT and 6-FDT) was tested and compared by using both chemical and chemoenzymatic approaches. **(A)** For ‘cold’ 2-FDT synthesis, we tested 3 different chemoenzymatic approaches, see the main text for further details. **(B)** Cold syntheses of both 4-*epi*-FDT and 6-FDT were tested from the corresponding mono-hydroxy precursors; corresponding *O*-triflate derivatives were synthesised in high yield (88% for 6-OTf-Tre(Ac)₇ and 84% for 4-OTf-Tre(Ac)₇). Subsequently the substitution of these triflate precursors yielded corresponding fluorinated sugars (6-FDT 60%, 4-*epi*-FDT 15%). These formed the basis for synthetic routes to corresponding ‘hot’ ¹⁸F variants (see

Figure S3). (C) ‘Cold’ synthesis of 3-FDT was attempted in two stages. In the first stage, precursor 3-fluoro-3-deoxy-D-glucose (3-FDG) was synthesised directly using DAST (to provide a reference) and also from the corresponding triflate precursor (to mimic putative radiosynthesis), as confirmed by ^1H NMR of reaction product (D). This 3-FDG was then used in attempted, one-pot chemoenzymatic syntheses of 3-FDT (2.5 mM scale in HEPES buffer solution, 50 mM HEPES buffer, pH 7.25, 30 °C with UDP-Glc(33 mM), hexokinase (5 mg/mL), ATP (8.3 mM), OtsA (1.3 mg/mL) and OtsB (3.4 mg/mL)). Progress of reaction was monitored by HPLC (HPLC chromatograms of this reaction are shown in (E)) but did not reveal effective conversion/separation in a manner concomitant with automated radiosynthesis. These were consistent with poor results from attempted synthesis of 3- ^{18}F]FDT (see below).



E

Time (min)	% FDG	% 2-FDT
50	1.5	98.5
115	5.4	94.6
urine (120)	4.2	95.8

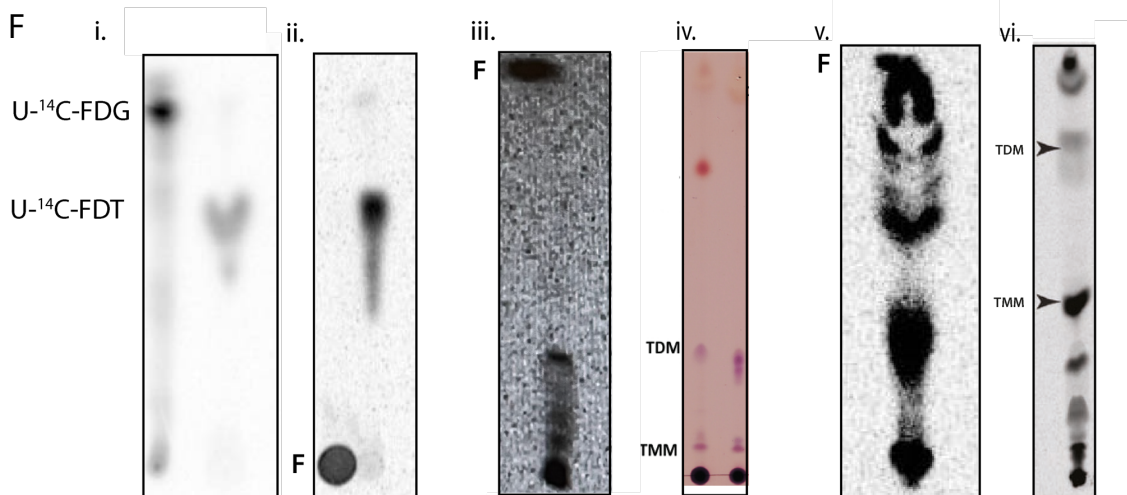


Figure S3. Comparison of Trehalose Analogs – Synthetic routes, Biodistribution/Biostability and Lipid Incorporation.

(A) Synthetic route to 6- ^{18}F FDT. (B) Synthetic route to 4-*epi*- ^{18}F FDT. (C) Concentration-time plot showing that 4-*epi*- ^{18}F FDT was metabolized to 4-*epi*-FDG within the typical clearance timeframe of a clinical imaging session with an ^{18}F probe. The all but final timepoints were from blood samples taken from the injected subject. The final timepoint represents the ^{18}F F-labeled constituents in the urine drawn from the bladder at the indicated time. (D) Concentration-time plot showing that 6- ^{18}F FDT was metabolized rapidly to 6-FDG within the typical timeframe of a clinical imaging session with a ^{18}F probe. (E) Tabular comparison of ^{18}F FDT metabolism timecourse, using essentially identical methods, illustrating that when ^{18}F was incorporated at the 2 position in 2- ^{18}F FDT / ^{18}F FDT was less subject to metabolism than when placed into the 6 or 4 position. (F) Radio-TLC analysis of the generation of $[\text{U-}^{14}\text{C}, ^{19}\text{F}]$ FDT (i reaction mixture; ii isolated, mobile phase 4:4:1 EtOAc:iPrOH:water) and its incorporation (iii, mobile phase 90:9:1 CHCl_3 :MeOH:water; v, mobile phase 75:25:4 CHCl_3 :MeOH) into *Mtb* lipids. These are compared with TLC run under similar conditions (iv, mobile phase 90:10:1 CHCl_3 :MeOH:water; vi, mobile phase 75:25:4 CHCl_3 :MeOH, respectively) taken from native lipid staining (Guallar-Garrido *et al.*²) and use of $[\text{U-}^{14}\text{C}]$ trehalose (Backus *et al.*³), respectively. F denotes fiducial marker for baseline or solvent front.

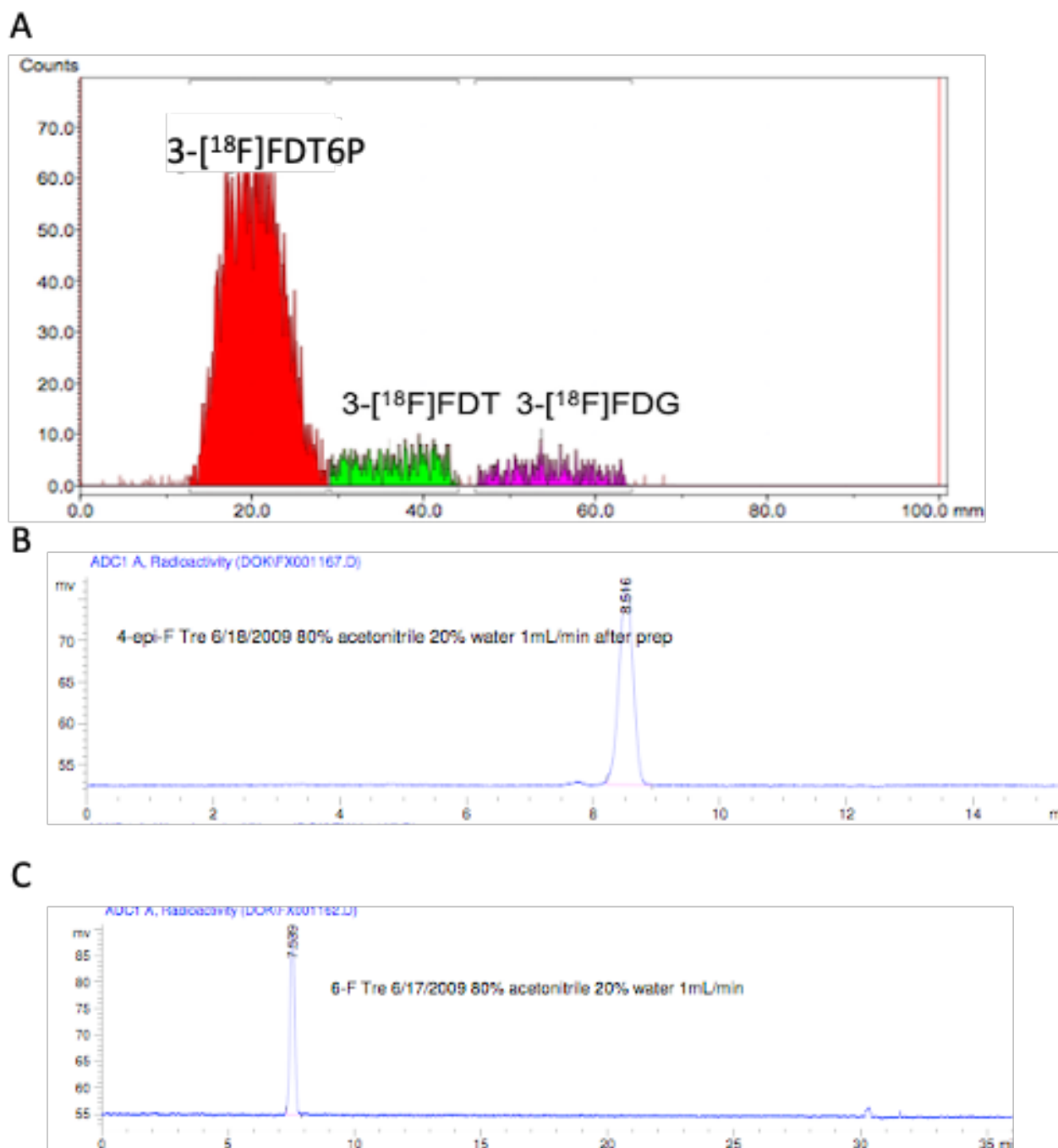


Figure S4. Chromatograms for the Radiosynthesis of ^{18}F FDT Analogs.

A) Radio-TLC chromatogram of 3- ^{18}F FDT synthesis from 3- ^{18}F FDG. Only 10% conversion to 3- ^{18}F FDT observed over 60 min ($R_f=0.31$; region 2), whereas 83% of the intermediate product 3- ^{18}F FDT6P was observed at the start ($R_f=0.0$; region 1) and around 6% of the starting material i.e. 3- ^{18}F FDG observed ($R_f=0.54$; region 3). **B)** Analytical radio HPLC chromatogram of 4-epi- ^{18}F FDT synthesis, showing full conversion to the desired

product with 9% yield. C) Analytical radio HPLC chromatogram of 6-[¹⁸F]FDT with 25 ± 3% overall yield (n=2)

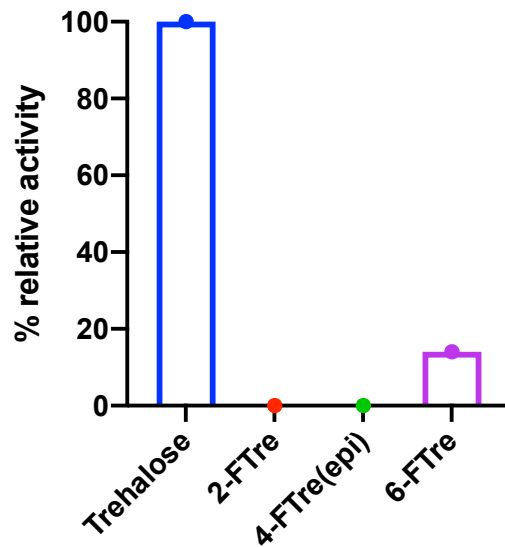


Figure S5. Trehalase Assays.

Substrates were prepared to a final concentration of 10 mM in 0.05 M citric acid, pH 5.7 including trehalase stock solution (0.3 units/mL). The reaction mixture was incubated at 37 °C for 60 minutes, and then quenched with an equal volume of 0.1 M Tris, pH 7.5. Released glucose was measured with glucose assay kit (see Methods).

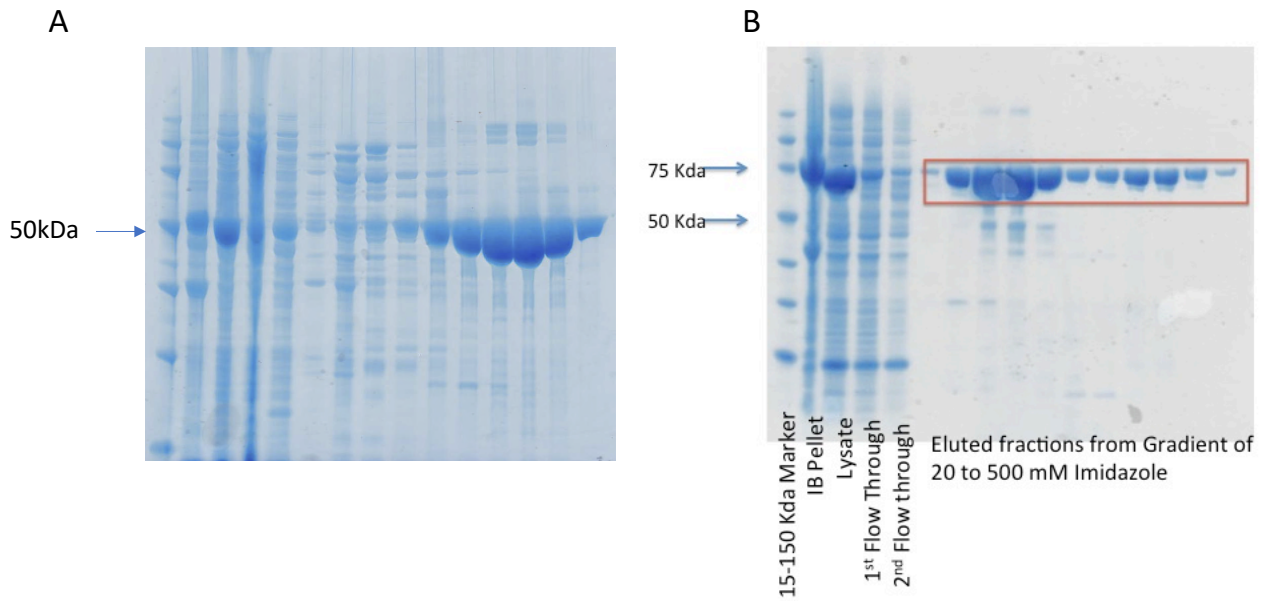


Figure S6. OtsA and OtsB *E. coli* Expression, Purification and Characterisation.

A) 10% Bis-Tris SDS PAGE Analysis of OtsA expression run in MOPS buffer at 200 V for 50 minutes. Analytes: 15-150 KDa Protein marker, IB pellet, Lysate, 1st two flow through, 2nd-11th eluted fractions. **B)** SDS-PAGE Analysis of His-MBP-OtsB protein expression.

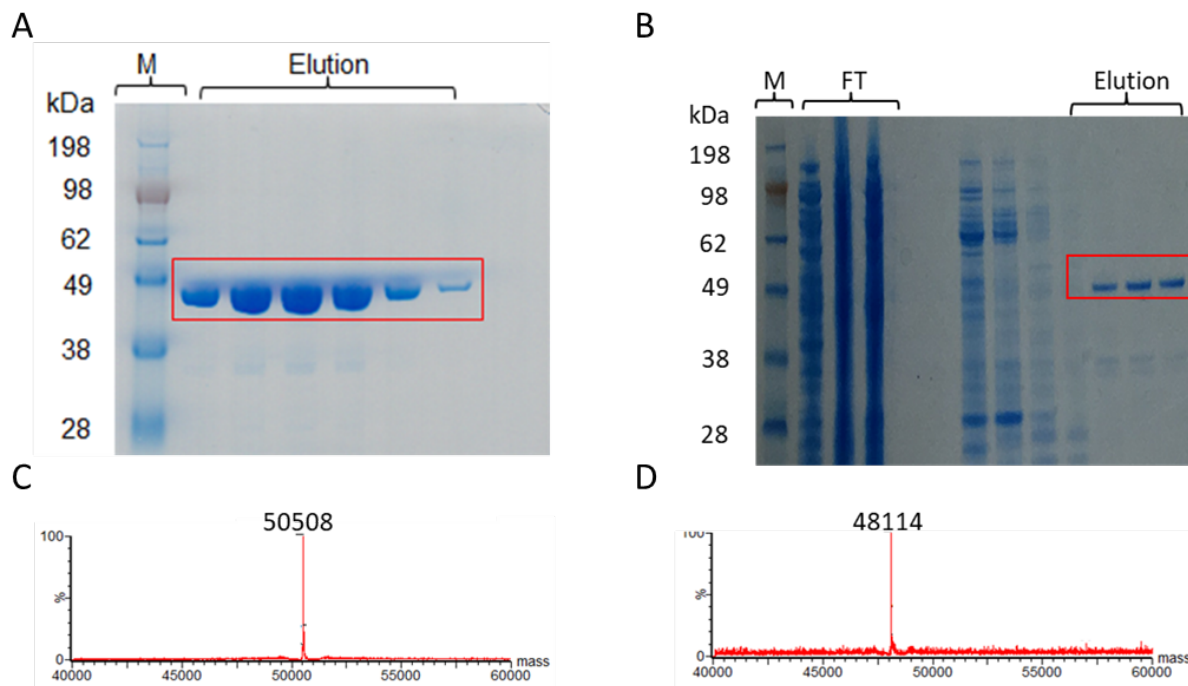


Figure S7. TreT *P. horikoshii* and TreT *T. tenax* Enzyme Characterization and Reaction Analysis.

Details of TreT homologue purification, characterisation and enzyme reactions. **A)** & **C)**.

TreT *P. horikoshii* enzyme characterisation by SDS-PAGE and LC-MS. Similarly **Figure B)**

& **D)** represent TreT *T. tenax* characterisation by SDS-PAGE and LC-MS.

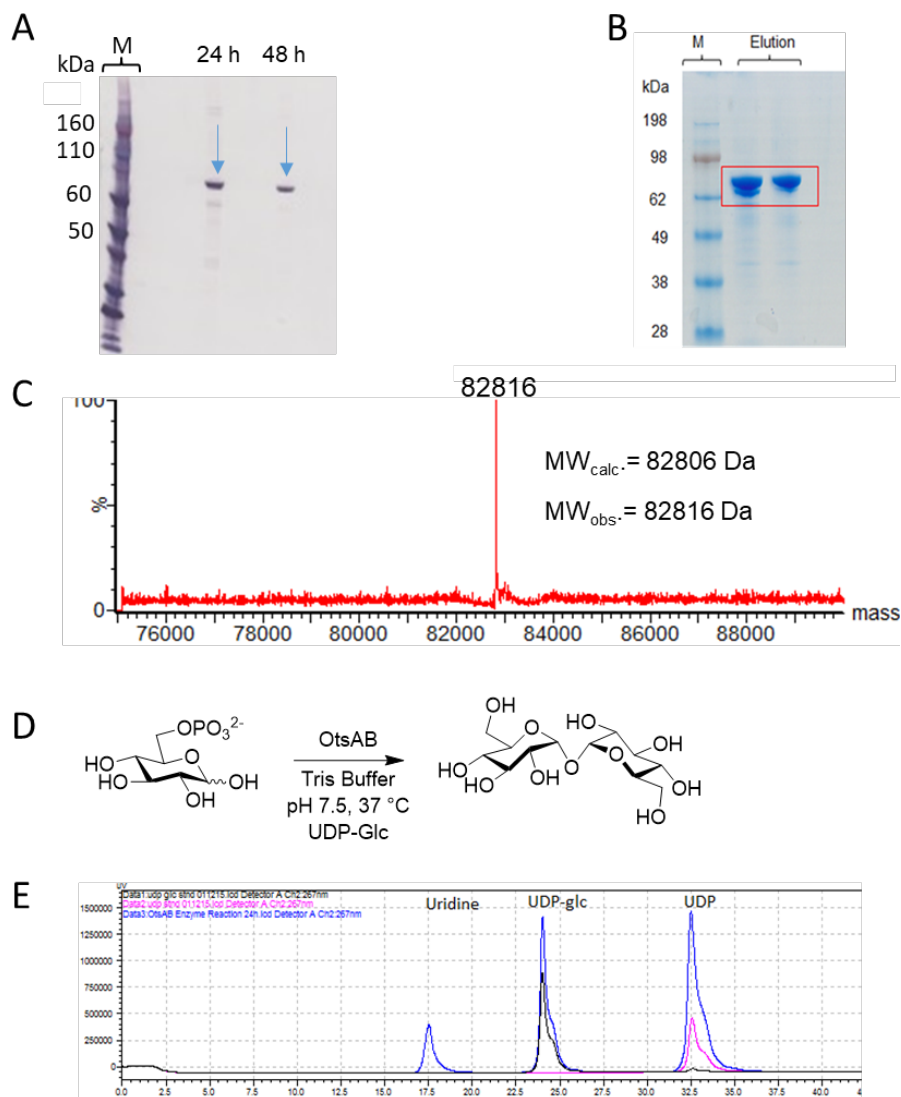


Figure S8. OtsAB fusion Enzyme Optimisation and Expression.

A) Small scale expression with different IPTG concentrations and OD₆₀₀, values. A His-tagged protein at the expected molecular weight was visualised by Western blotting after probing with anti-His monoclonal antibody after 24 h and 48 h of incubation at 15 °C. IPTG concentration of 0.5 mM and OD₆₀₀ of 0.4 were the relatively best conditions as compared to IPTG concentration 1 mM and OD₆₀₀ 0.6. B) SDS-PAGE characterisation of the large scale (1L) expression of protein. C) LC-MS characterisation of OtsAB fusion enzyme where the expected mass after N-terminus Met cleavage is observed D) Reaction scheme for the enzyme reaction of OtsAB fusion enzyme. and E). HPLC chromatogram of the glucose-6-phosphate

reaction in the presence of UDP-Glc and OtsAB fusion enzyme. The reaction monitoring by HPLC confirmed the UDP release which is one of the product of this enzyme catalysis.

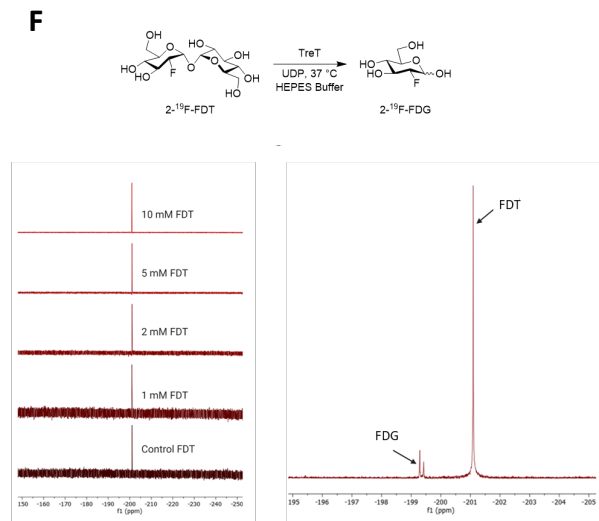
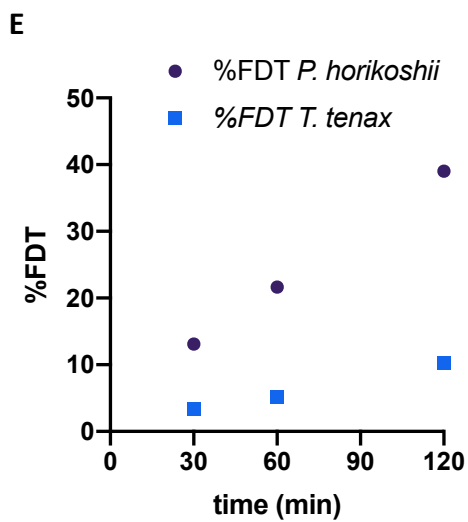
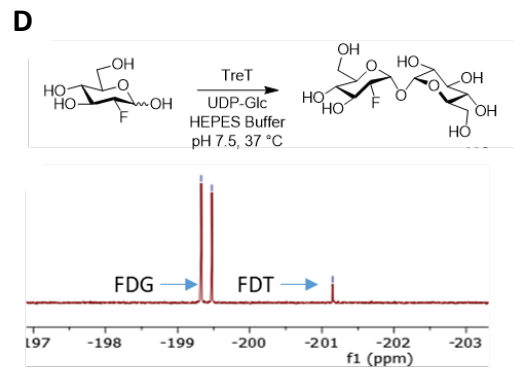
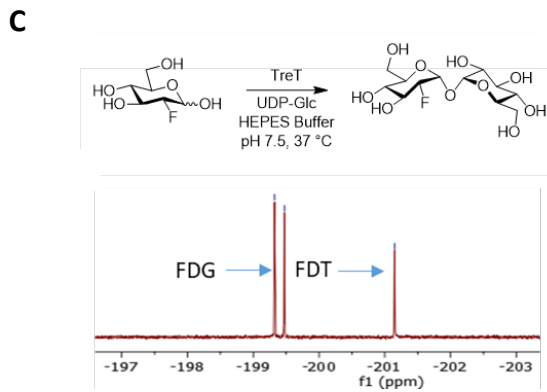
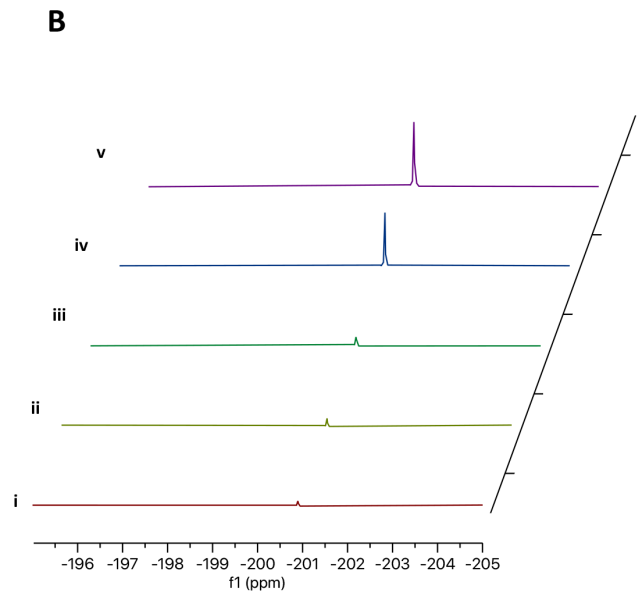
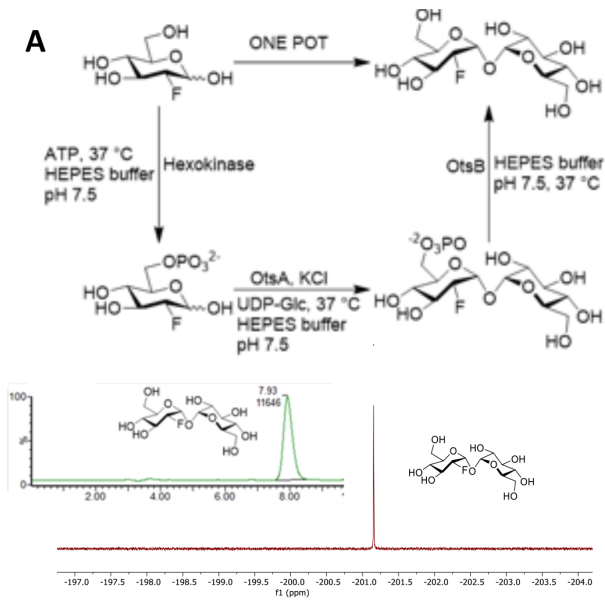


Figure S9. Comparison of different biocatalytic systems for the synthesis of ‘cold’ FDT on a small scale.

Routes as described in **Figure 1A**. **(A)** Full conversion of substrate FDG (5.5 mM) into FDT within 45 min as shown by ^{19}F NMR spectroscopy and LC-MS integrated chromatogram (inset) of [^{19}F]FDT reaction sample, showing the formation of the product at rt 7.93 min. **(B)** The reaction was complete on a range of FDG substrate amounts: **i)** 436 μM ; **ii)** 581 μM ; **iii)** 1.162 mM; **iv)** 2.324 mM & **v)** 4.648 mM, all with full conversion as judged by ^{19}F NMR spectroscopy to the product FDT within 45 min. **(C & D)** Conversion using two TreT homologue enzyme from *P. horikoshii* and *T. tenax*, respectively (shown at 10 mM) gave an estimated conversion for *P. horikoshii* was 40% and for the *T. tenax* ~ 10% as judged by ^{19}F NMR spectra (shown) when reactions were carried out under the same conditions over 2 h. **(E)** Time course of the reactions shown in **(C, D)** using ^{19}F NMR. **(F)** The *P. horkoshii* TreT enzyme is reversible converting FDT back to FDG, as shown when tested at higher FDT substrate concentrations (20 mM) and higher UDP concentration (80 mM); less ‘reversion’ was observed under the same conditions at lower UDP (20 mM) and FDT concentrations (1-10 mM).

A

OtsA <i>E. coli</i> N-terminus		OtsA <i>E. coli</i> C-terminus	
DNA Sequence	ATGAGTCGTTTAGTCGTAGTATCTAACCGGATTGCA CCACCAGACGAGCACGCCGCCAGTGCCGGTGGCCTT GCCGTTGGCATACTGGGGGCACTGAAAGCCGCAGGC GGACTGTGGTTTGGCTGGAGTGGTGAACAGGGAA	DNA Sequence	CTGGCGGAACGTATTTCCCGTCATGCAGAAATGCTGGA CGTTATCGTGAAAAACGATATTAACCACTGGCAGGAGT GCTTCATTAGCGACCTAAAGCAGATAGTTCCGCGAAGC CGGAAAGCCAGCAGCGCGATAAAGTTGCTACCTTTC
AA Sequence	GAGGATCAGCCGCTAAAAAAGGTGAAAAAAGGTAAC MSRLVVVS NRIAPPDEHAASAGGLAVGILGALKAAG GLWFGWSGETGNEDQPLKKVKKGN	AA Sequence	LAERISRHAEM LDVIVKNDINHWQECFISDLKQIVPRS AESQQRDKVATFPKLALEHHHHHH-
OtsA <i>Sf9</i> N-terminus		OtsA <i>Sf9</i> C-terminus	
DNA Sequence	ATGAGCCGCTGGTCTGGTGTCCAACCGCATCGCTCCCC TGACGAACATGCCGCTAGCGCTGGTGGTCTCGTGTGGTA TCCTCGGCGCTCTGAAGGCTGCCGGTGGTCTGTGGTTTGGC TGGAGTGGTGAACAGGGAATGAGGATCAGCCGCTAAAAA GGTGAAAAAA GGCAAC	DNA Sequence	CTGGCTGAACGTATCTCCCGCCATGCCGAGATGCTGG ACGTCATCGTGAAGAACGATATTAACCACTGGCAGGA GTGCTTCATTAGCGACCTAAAGCAGATAGTTCCGCGA AGCGCTGAGAGCCAGCAGCGCGACAAGGTGGCCACCT TCCCTAAGCTGGCCCTGGAGCACCAT CACCACCACCGCG
AA Sequence	MSRLVVVS NRIAPPDEHAASAGGLAVGILGALKAAGGLWFG WSGETGNEDQPLKKVKKGN	AA Sequence	LAERISRHAEM LDVIVKNDINHWQECFISDLKQIVPR SAESQQRDKVATFPKLALEHHHHHH

B

OtsB <i>E. coli</i> N-terminus		OtsB <i>E. coli</i> C-terminus	
DNA Sequence	ATGGGCAGCAGCCACCACCACCACCACGGCACCAGAC CGAGGAGGGCAAGCTGGTGTCTGGATCAACGGCGACAAGG GCTACAACGGCCTGGCCGAGGTGGGCAAGAAGTTCGAGAAG GACACCGGCATCAAGGTGACCGTGGAGCACCCCGACAAGCT GGAGGAGAAGTTCCCCTGGCCGAGATCAAGCCCCACCCC ACCAGGTGGTGGTG	DNA Sequence	GGCTTCGCCGTGGTGAACAGGCTGGGCGGCATGAGCG TGAAGATCGGCACCGGCCACCCAGGCCAGCTGGAG GCTGGCCGGCGTGCCCGACGTGTGGAGCTGGCTGGAG ATGATCACCACC GCCCTGCAGCAGAAGAGGGAGAACA ACAGGAGCGACACTACGAGAGCTTACGAGGAGCAT C
AA Sequence	MGSSHHHHH GTKEEGLVIWINGDRGYNGLAEVGGKFEK DTGIKVTVEHPDKLEEKFLAEIKPHPDQVVV	AA Sequence	GFAV VNRLGGMSVKIGTGATQASWRLAGVPDVWSWLE MITTALQKRENNRSDDYESFSRSI
OtsB <i>Sf9</i> N-terminus		OtsB <i>Sf9</i> C-terminus	
DNA Sequence	ATGAGCTACTACCACCACCACCACCACGACTACGACAT CCCCACCACCGAGAACCTGTACTTCCAGGGCCACATGGTGA CCGAGCCCTGACCGAGACCCCGAGCTGAGCGCCAAGTAC GCCTGGTTCTTCGACCTGGACGGCACCCCTGGCCGAGATCAA GCCCCACCCGACCAGGTGGTGGTG	DNA Sequence	GGCTTCGCCGTGGTGAACAGGCTGGGCGGCATGAGCG TGAAGATCGGCACCGGCCACCCAGGCCAGCTGGAG GCTGGCCGGCGTGCCCGACGTGTGGAGCTGGCTGGAG ATGATCACCACC GCCCTGCAGCAGAAGAGGGAGAACA ACAGGAGCGACACTACGAGAGCTTACGAGGAGCAT C
AA Sequence	SYHHHHHH HDYDIPPTENLYFQGHMVTEPLTETPELSAKYA WFFDLDTLAEIKPHPDQVVV	AA Sequence	GFAV VNRLGGMSVKIGTGATQASWRLAGVPDVWSWLE MITTALQKRENNRSDDYESFSRSI

Figure S10. Sequence Comparison of OtsA and OtsB Sources.

A) Comparison of N-terminus and C-terminus residues of OtsA sequences from both *E. coli* and *Sf9*. **B)** Comparison of N-terminus and C-terminus residues of OtsB sequences from both *E. coli* and *Sf9*.

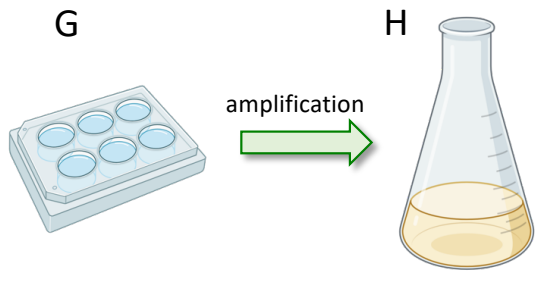
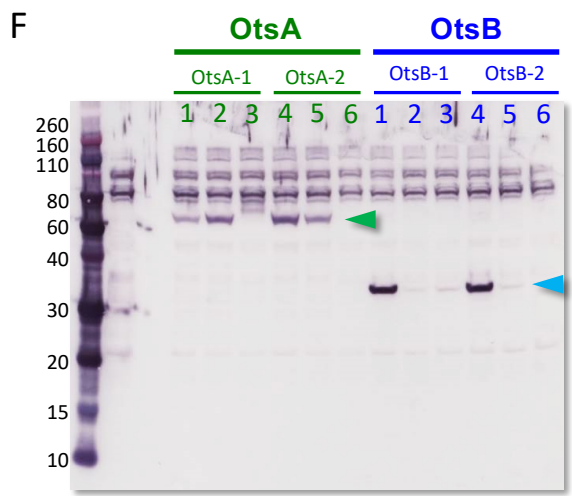
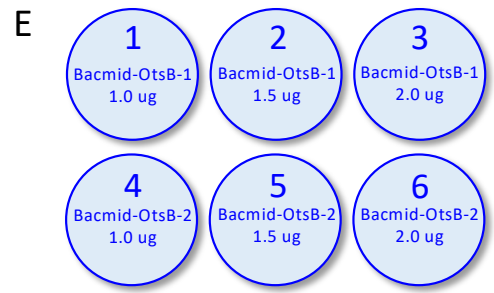
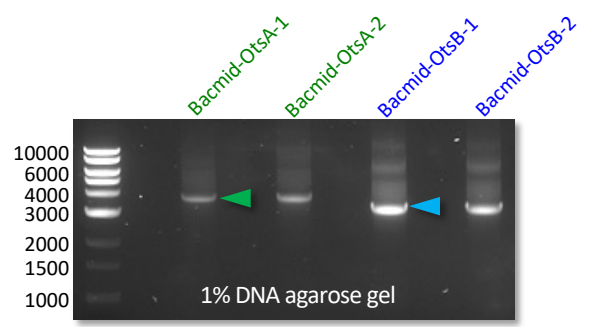
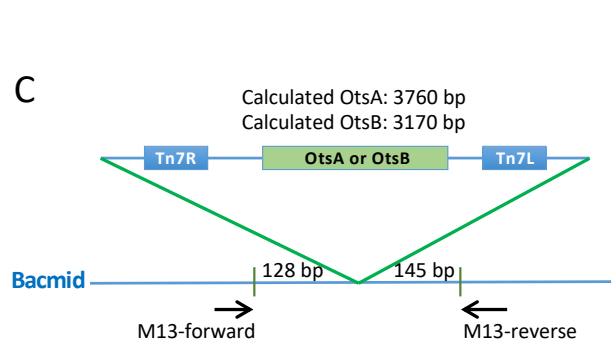
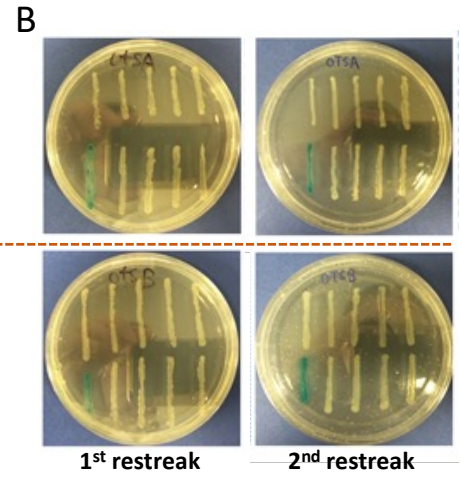
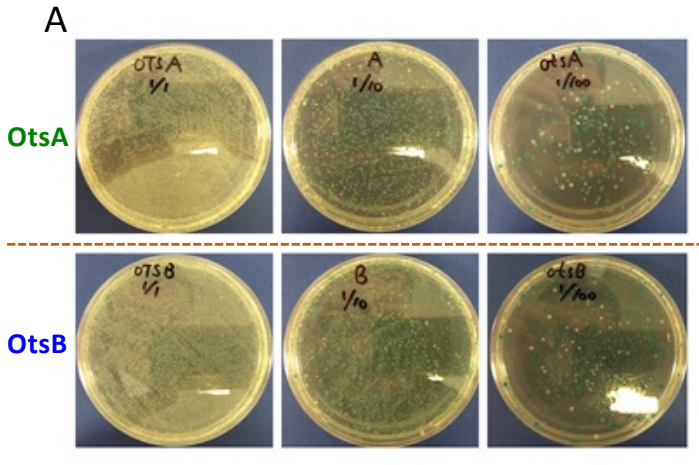


Figure S11. Schematic of the insect cell (Sf9) expression of OtsA^{Sf} and OtsB^{Sf}.

A) Blue/white selection screening of OtsA and OtsB recombinant bacmid DNAs. **B)** Phenotype confirmation by streaking and re-streaking the selected colonies from A). **C)** PCR analysis to confirm the insertion of *otsA* and *otsB* gene into bacmid DNA. **D & E)** Generation of P1 baculovirus for OtsA and OtsB enzymes by Sf9 cell transfection with different amounts of bacmid DNA in a 6-well plate. **F)** Western blot analysis using *anti*-His tag antibody to characterize the expression of OtsA and OtsB proteins based on the cell pellets obtained during the harvest of their P1 baculovirus, respectively. **G & H)** Amplification of the P1 baculovirus of OtsA and OtsB to generate the P2 baculovirus stock. Panels G,H created with [BioRender.com](https://www.biorender.com), released under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International license.

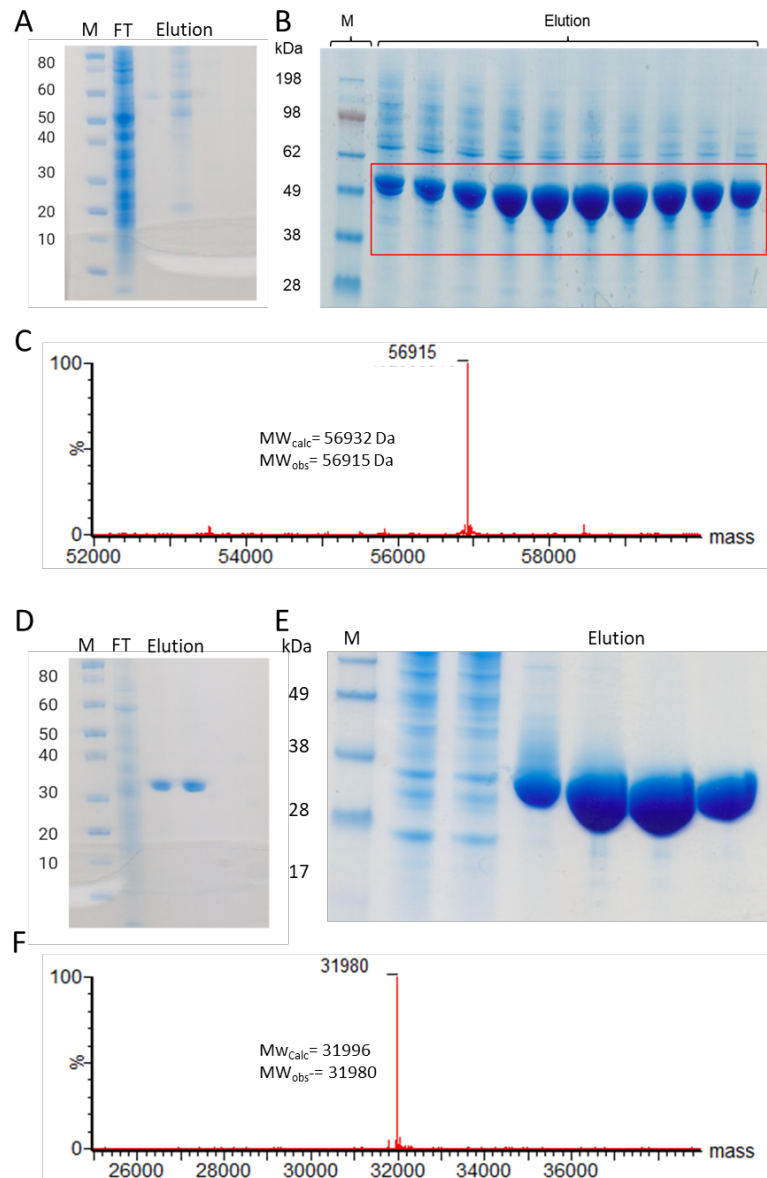


Figure S12. Small and Large scale Expression of OtsA and OtsB enzymes from Insect cells.

A) SDS-PAGE characterization of OtsA enzyme from the pellet of 50 mL culture. **B)** SDS-PAGE characterization of OtsA enzyme from 1 L culture. **C)** LC-MS characterization of OtsA enzyme. **D)** SDS-PAGE characterization of OtsB enzyme from the pellet of 50 mL culture. **E)** SDS-PAGE characterization of OtsB enzyme from the 1L scale expression culture. **F)** LC-MS characterization of OtsB enzyme.

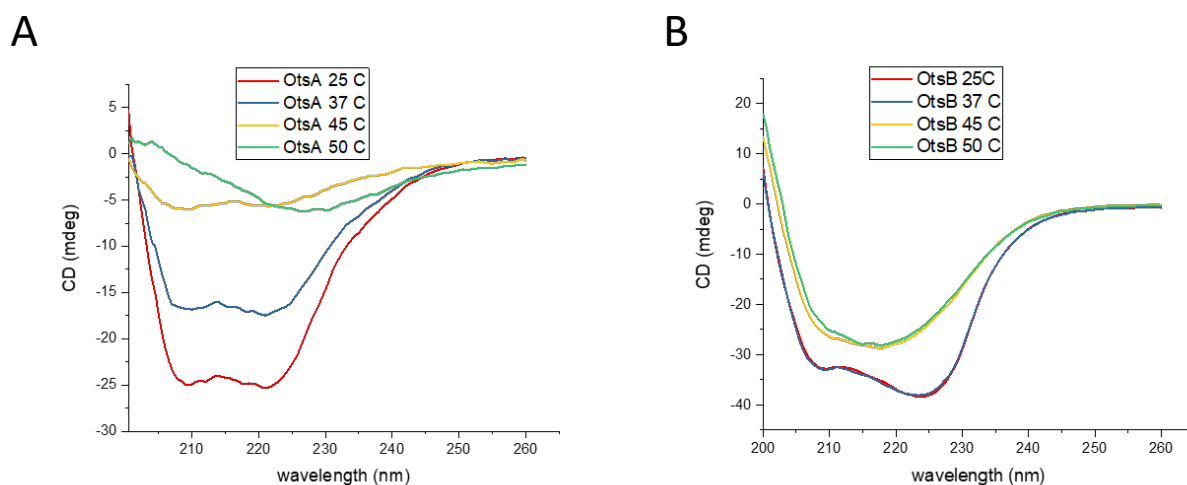


Figure S13. CD spectra and Stability Assessment of OtsA and OtsB enzymes.

A). CD spectra of OtsA enzyme incubated at 25 °C, 37 °C, 45 °C and 50 °C for 1h. **B).** CD spectra of OtsB enzyme at 25 °C, 37 °C, 45 °C and 50 °C under the same conditions. Both enzymes maintained secondary structure at 25 °C and 37 °C, however, at higher temperatures secondary structure of protein was lost.

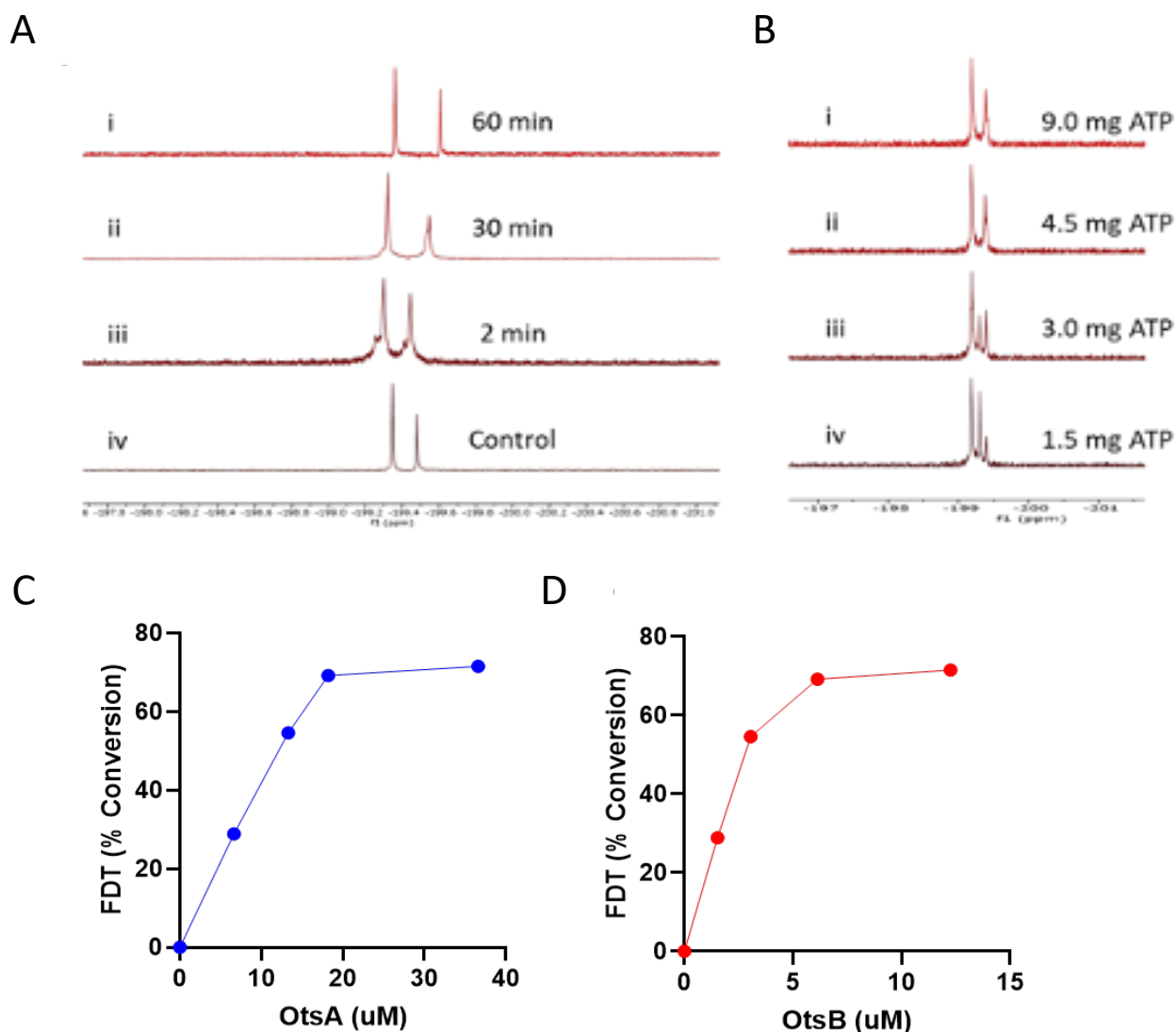


Figure S14. Optimisation Screen during Enzyme Reaction Scale-up.

A, B) Testing the optimal amount of hexokinase and ATP needed for full conversion of FDG to FDG6P. **A).** Reaction monitoring from FDG to FDG6P using 5 mg hexokinase. Reaction completed after 60 min. **B).** Small scale reactions of FDG with ATP to determine the optimal amount of ATP needed for conversion of FDG to FDG6P. The amount of FDG was fixed at 5.6 mM or 1 mg, whereas ATP varied from 2.7 mM (1.5 mg), 5.4 mM (3 mg), 8.1 mM (4.5 mg) and 16.2 mM (9 mg). In conditions i and ii where concentration of ATP was higher than FDG, full conversion to FDG6P observed by ^{19}F -NMR spectroscopy.

C, D) OtsA and OtsB enzyme optimum concentration determination. For optimum enzyme concentration determination, reaction with fixed substrate concentrations and all the other parameters incubated with varying enzyme(s) concentrations. **C).** OtsA concentrations varied from 6.65 μM to 36.6 μM . Optimum concentration of the enzyme was found to be 18.2 μM . **D).** OtsB concentrations varied from 1.53 μM to 12.26 μM . Optimum concentration was 6.1 μM .

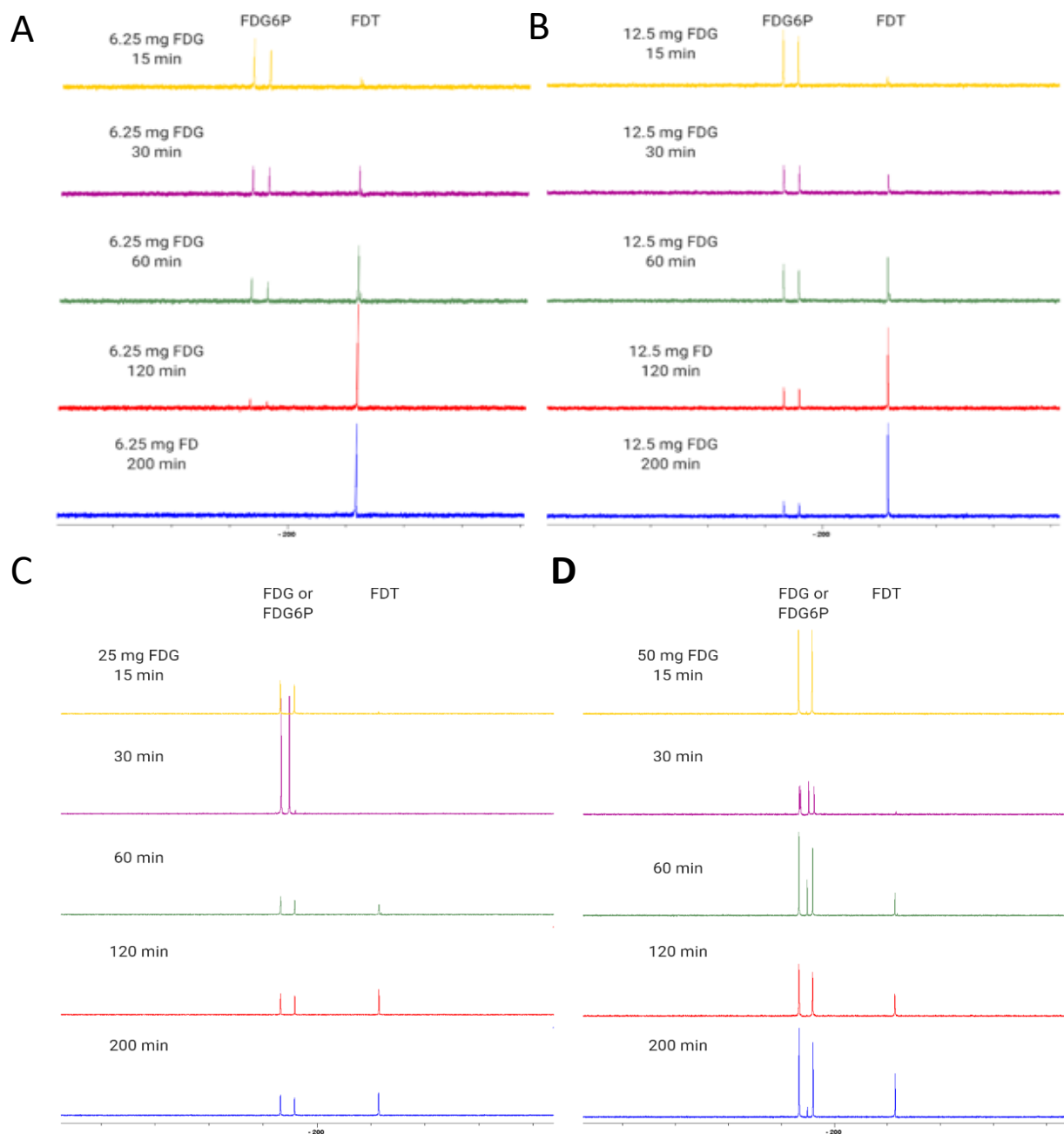


Figure S15. [¹⁹F]FDG Reaction Optimisation and Monitoring by ¹⁹F-NMR spectroscopy.

A). 6.25 mg FDG reaction catalysis by hexokinase, OtsA and OtsB. Samples were taken 15 min, 30 min, 60 min, 120 min and 200 min post reaction and reaction was more than 95% complete after 2 h. **B).** 12.5 mg FDG sample with similar monitoring time as above. After 3 h, there was 10% of FDG6P remained uncatalysed. **C).** 25 mg FDG sample catalysed and reaction monitored as A, after 3h, 20 min there was about 45% intermediate remained uncatalysed and **D).** 50 mg FDG sample catalysed and analysed as above, the conversion was 30% after 3h, 20 min. The glycosylation reaction was the limiting step and the amount of UDP-Glc was kept constant at 30 mM which was significantly less for 25 mg FDG substrate and 50 mg FDG substrate.

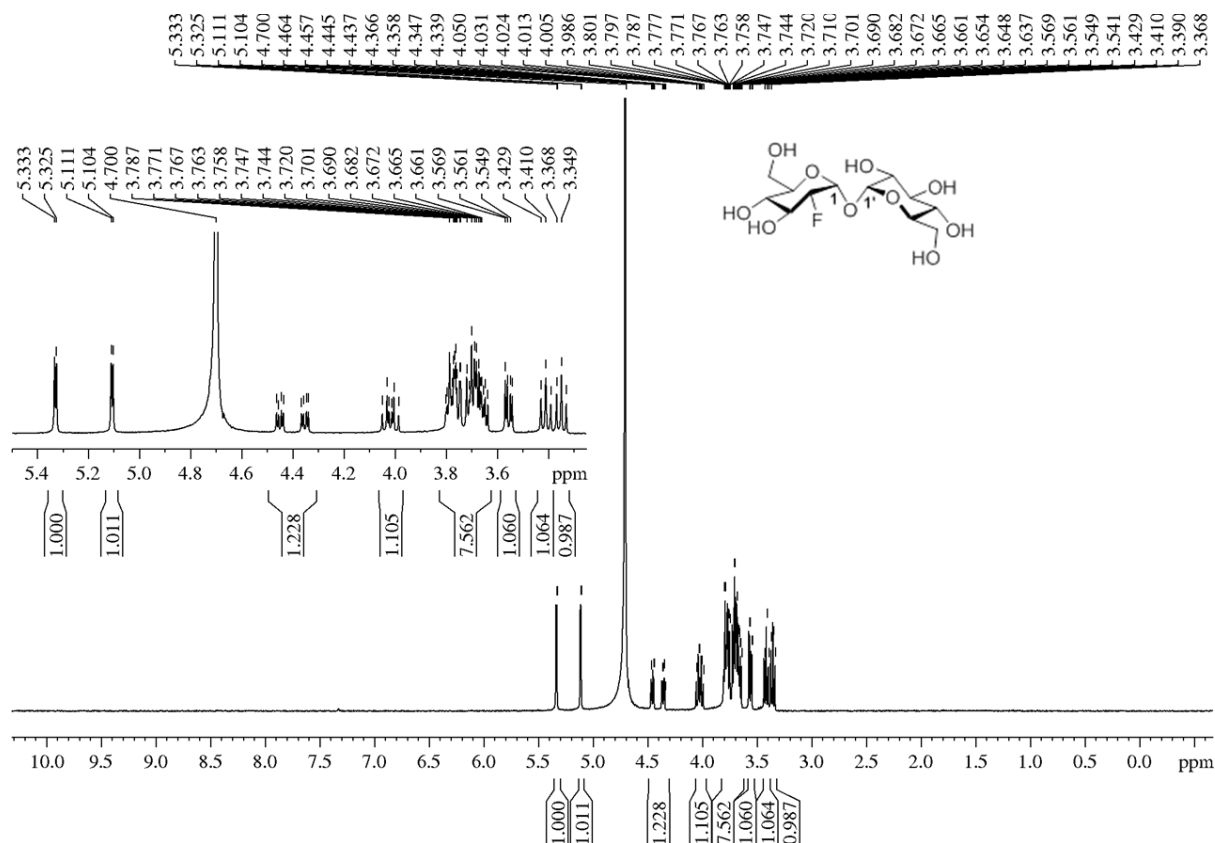


Figure S16. ^1H NMR spectrum of $[^{19}\text{F}]$ -FDT

Frequency [MHz]	500.13
SW [ppm]	19.9947
Origin	AVX500
Nucleus	^1H
NS	16
Solvent	D_2O
Temperature (degree C)	25

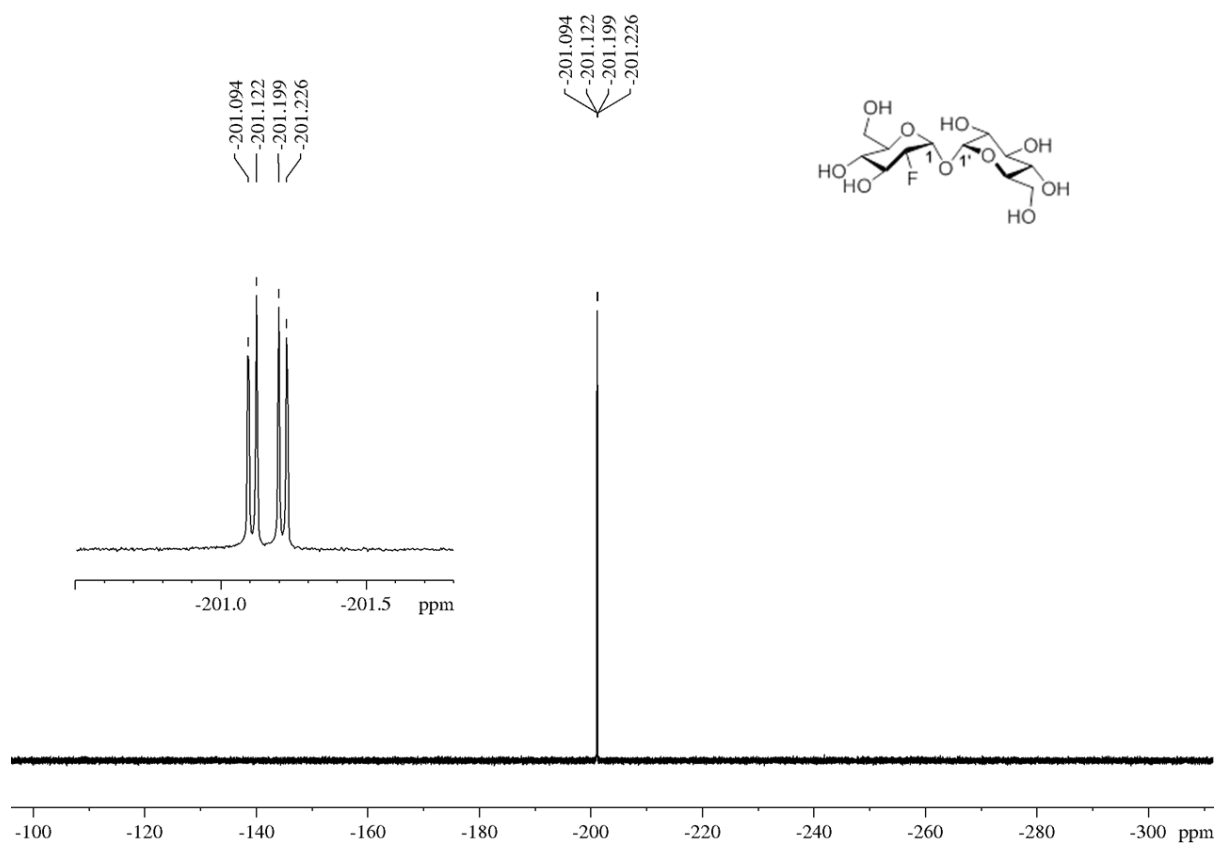


Figure S17. ^{19}F (^1H coupled) NMR spectrum of $[^{19}\text{F}]$ -FDT

Frequency [MHz]	470.13
SW [ppm]	241.5235
Origin	AVX500
Nucleus	^{19}F
NS	256
Solvent	D_2O
Temperature (degree C)	25

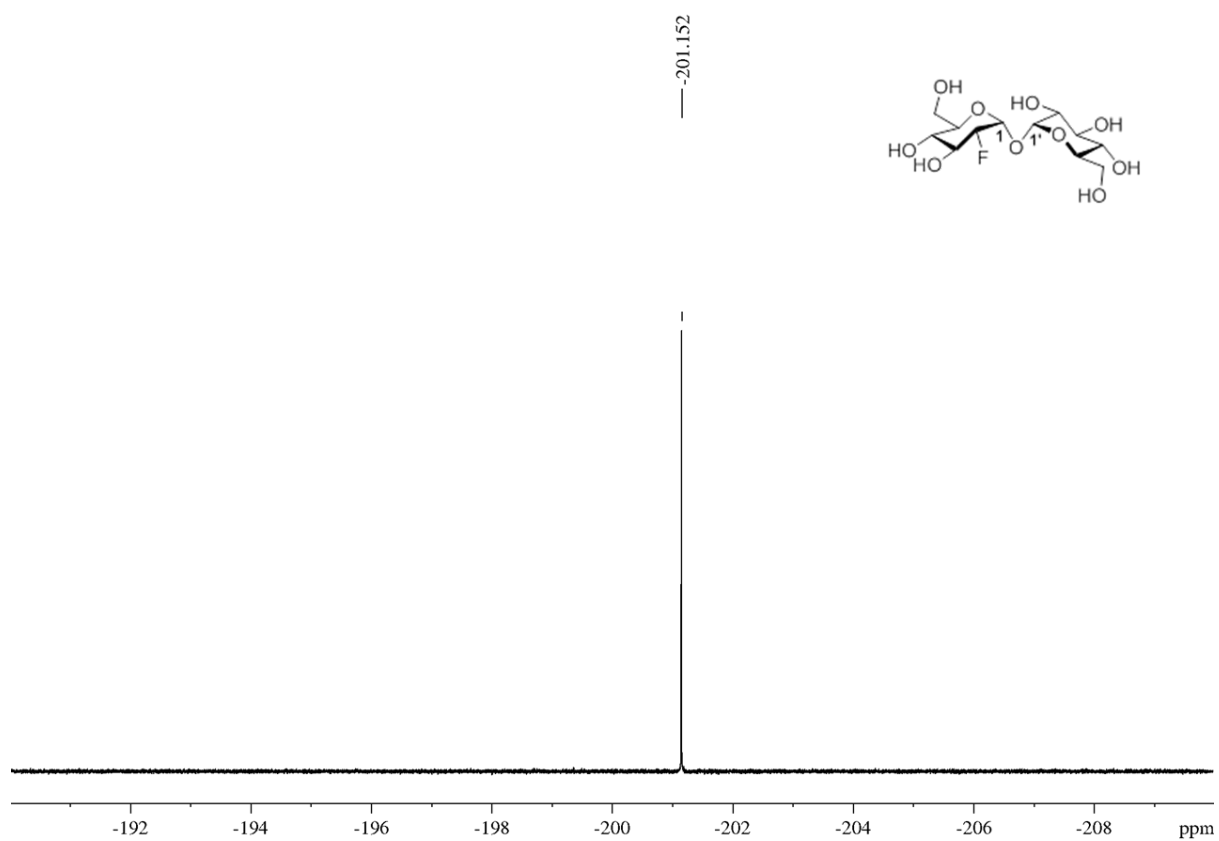


Figure S18. ^{19}F (^1H decoupled) NMR spectrum of [19F]- FDT

Frequency [MHz]	470.13
SW [ppm]	19.9756
Origin	AVX500
Nucleus	^{19}F
NS	32
Solvent	D_2O
D1 (sec)	10
Temperature (degree C)	25

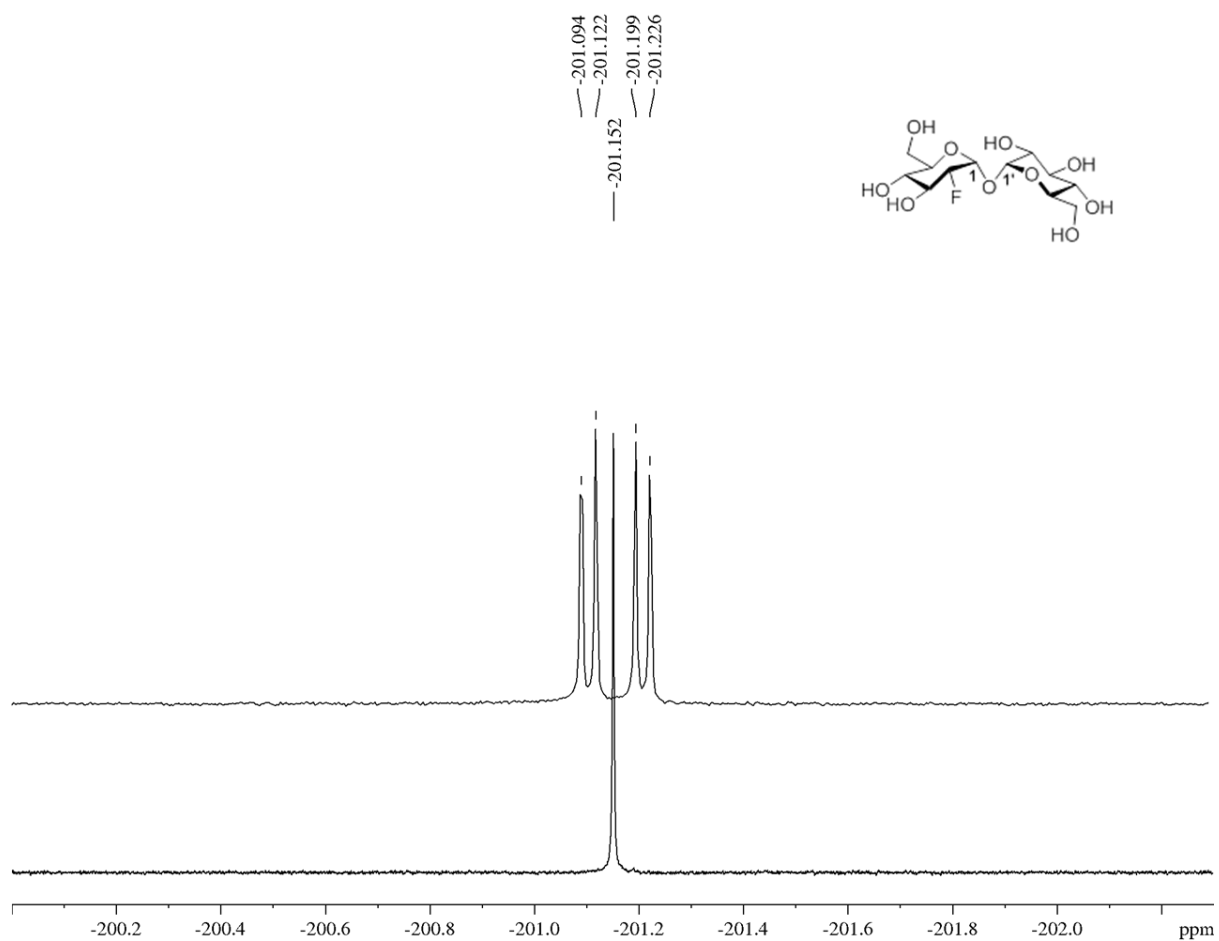


Figure S19. ^{19}F and $^{19}\text{F}(^1\text{H})$ Coupled NMR spectrum of $[^{19}\text{F}]$ -FDT.

Both coupled and decoupled spectra are stacked together.

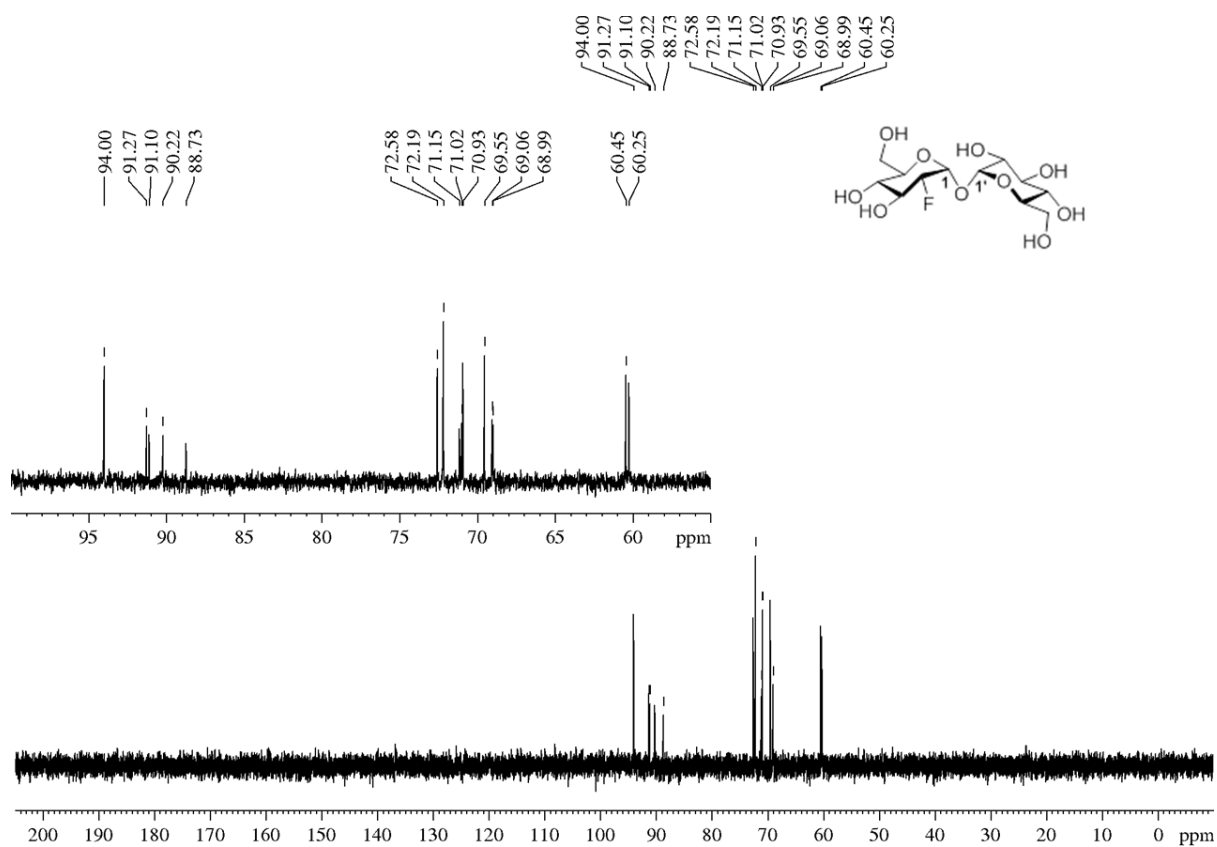


Figure S20. ^{13}C NMR spectrum of ^{19}F -FDT

Frequency [MHz]	126
SW [ppm]	236.64
Origin	AVX500
Nucleus	^{13}C
NS	3072
Solvent	D ₂ O
D1 (sec)	2
Temperature (degree C)	25

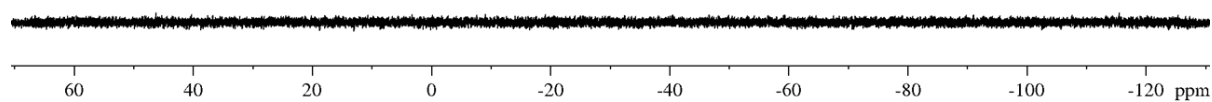
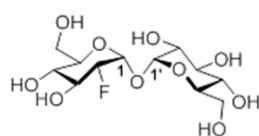


Figure S21. ^{31}P (^1H Decoupled) NMR Spectrum of [^{19}F]-FDT.

No signal for either reaction intermediates nor reagents used that contain ^{31}P nuclei.

Frequency [MHz]	202
SW [ppm]	201.3377
Origin	AVX500
Nucleus	^{31}P
NS	64
Solvent	D_2O
D1 (sec)	
Temperature (degree C)	25

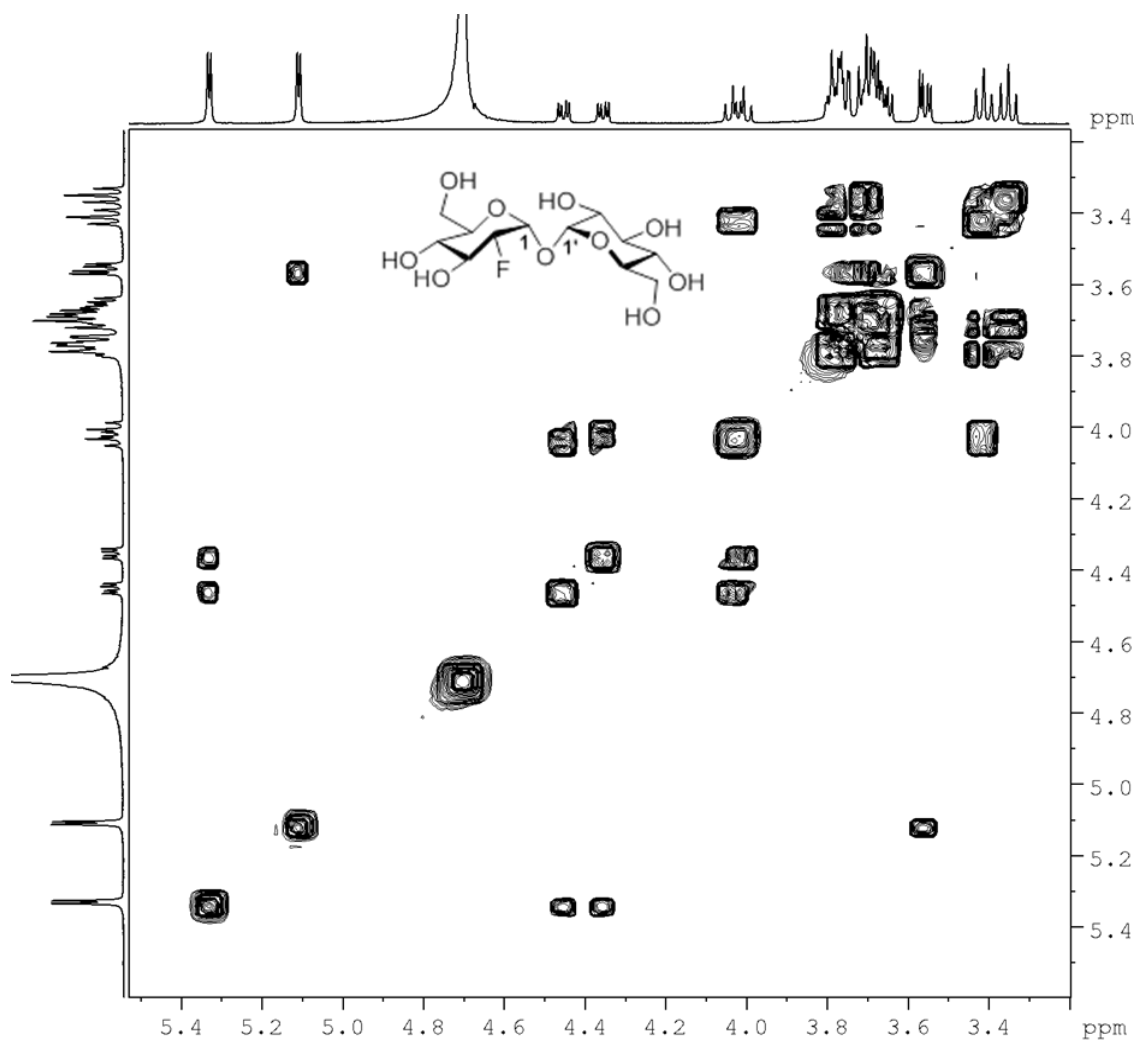


Figure S22. COSY spectrum of [¹⁹F]-FDT.

¹H-¹H COSY (Correlated Spectroscopy).

Frequency [MHz]	500
SW [ppm]	12.0161
Origin	AVX500
Nucleus	¹ H
NS	8
Solvent	D ₂ O

D1 (sec) 2

Temperature (degree C) 25

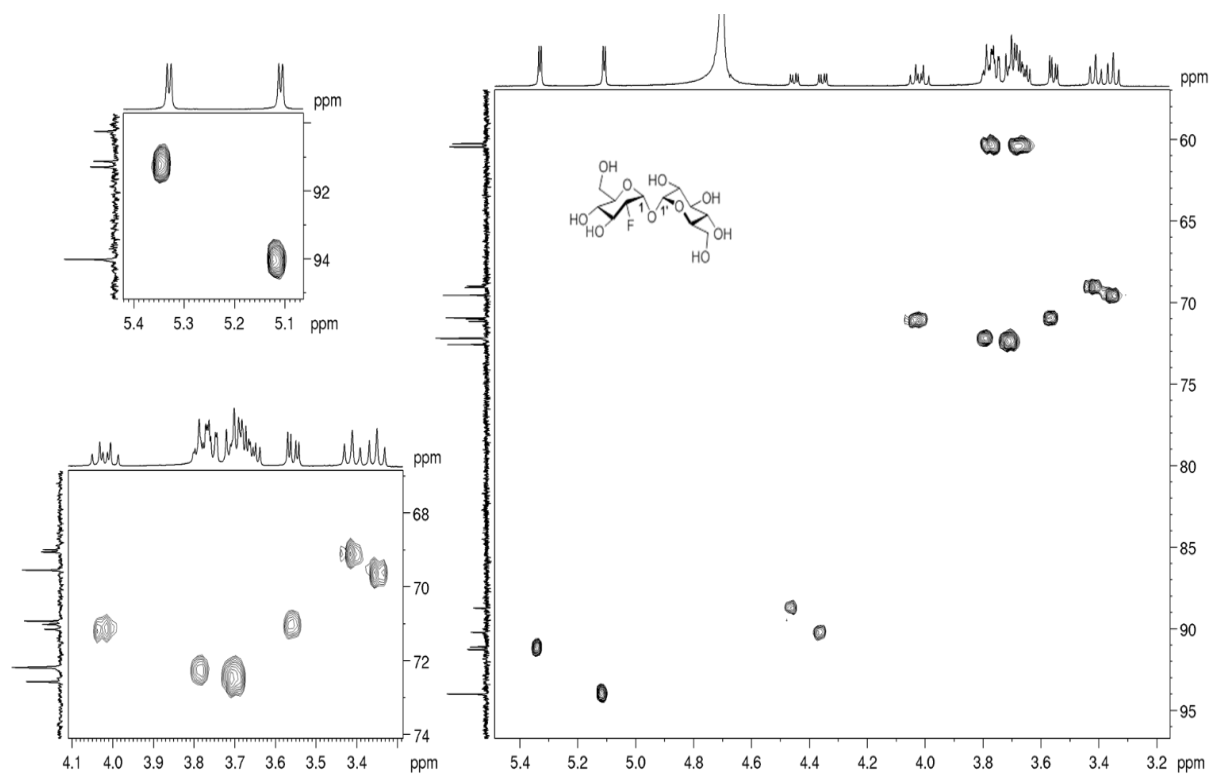
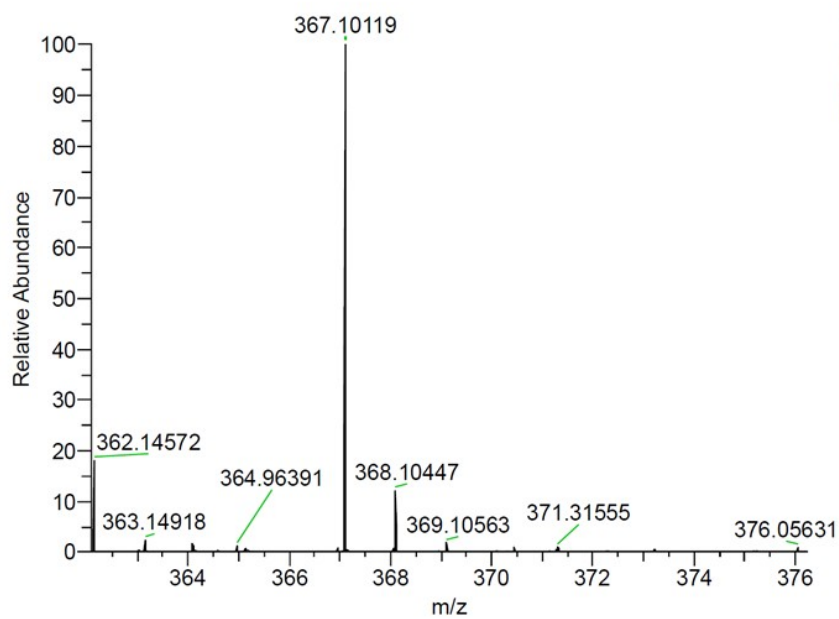


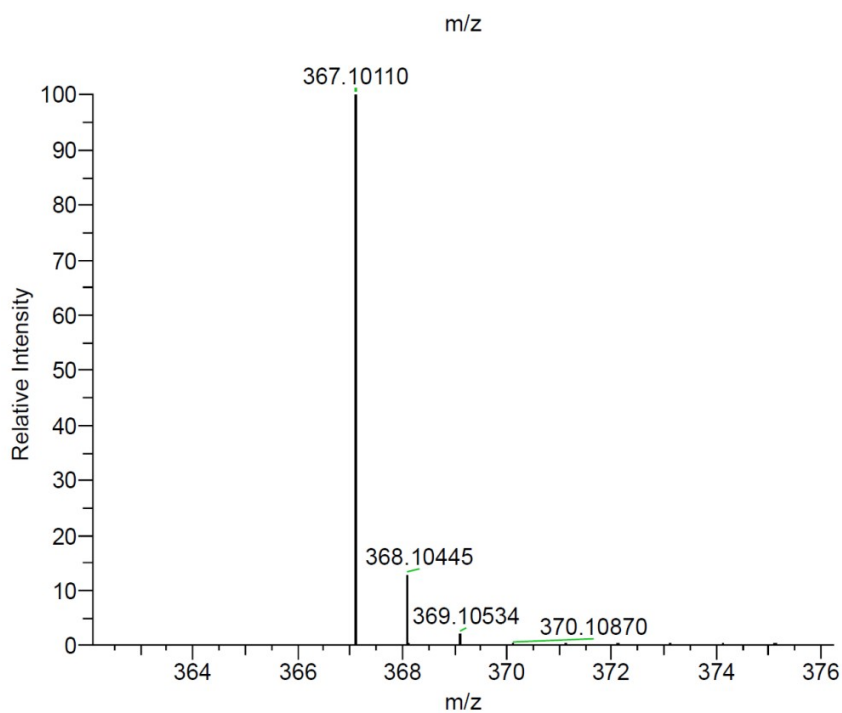
Figure S23. HSQC Spectrum of [¹⁹F]-FDT.

Heteronuclear Single Quantum Coherence experiment was used to determine proton-carbon single bond correlations. In the above spectrum ¹³C NMR spectrum is on the Y-axis whereas ¹H NMR spectrum is shown on the X-axis.

Frequency [MHz]	¹ H 500 & ¹³ C 126
SW [ppm]	12.0161
Origin	AVX500
Nucleus	¹ H – ¹³ C
NS	8
Solvent	D ₂ O
D1 (sec)	1
Temperature (degree C)	25



NL: 1.79E6
ESI74212 #14-27 RT: 0.17-0.31 AV: 7 NL:
1.38E+007
T: FTMS {1,1} + p ESI Full lock ms
[80.00-1600.00]



NL: 8.56E5
C12H21O10F1Na1: C₁₂ H₂₁ O₁₀ F Na
Chrg 1 R: 1000000 Res. Pwr. @FWHM

Figure S24. ESI-MS Spectrum of [¹⁹F]- FDT.

40 µg of [¹⁹F]- FDT Sample was dissolved in 1 mL 50:50 methanol de-ionised water and the accurate mass of the sample was determined. The instrument is accurate up to 4 decimal points.

The sample was run on a Thermo Orbitrap Exactive mass spectrometer calibrated with standard calibration solutions that included MRFA (L-methionyl-arginyl-phenylalanyl-alanine acetate.xH₂O), Ultramark 1621, caffeine and glacial acetic acid.

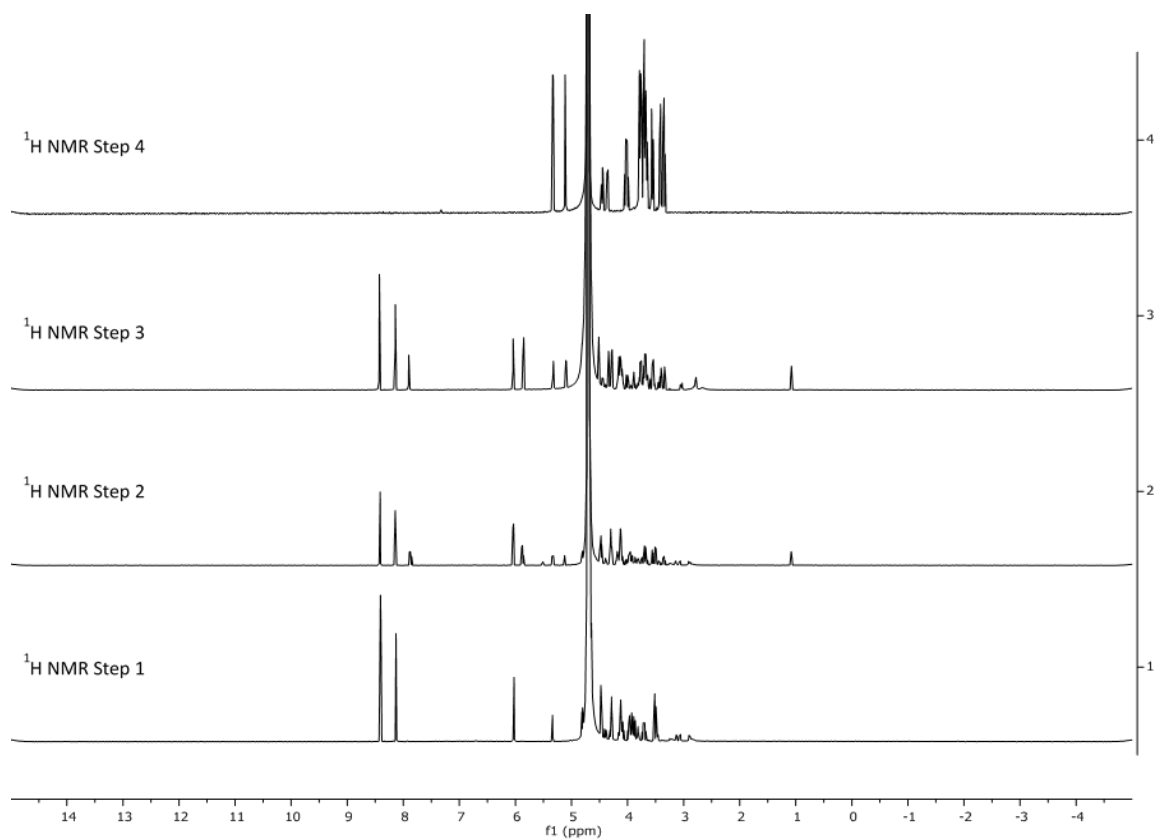


Figure S25. ^1H -NMR (500 MHz) spectra of crude reaction mixtures during one-pot synthesis and purified product.

Step 1 involves the conversion of **FDG** to **FDG6P**; Step 2 represents conversion of **FDG6P** to **FDT6P**; Step 3 shows the conversion of **FDT6P** to ^{19}F -**FDT**. Step 4 is for the purified final batch of ^{19}F -**FDT**. D_2O was used as solvent. For all crude reactions, 20 μL sample was diluted in 500 μL of D_2O ; whereas for the purified sample, 2.3 mg of ^{19}F -**FDT** was dissolved in 500 μL of D_2O .

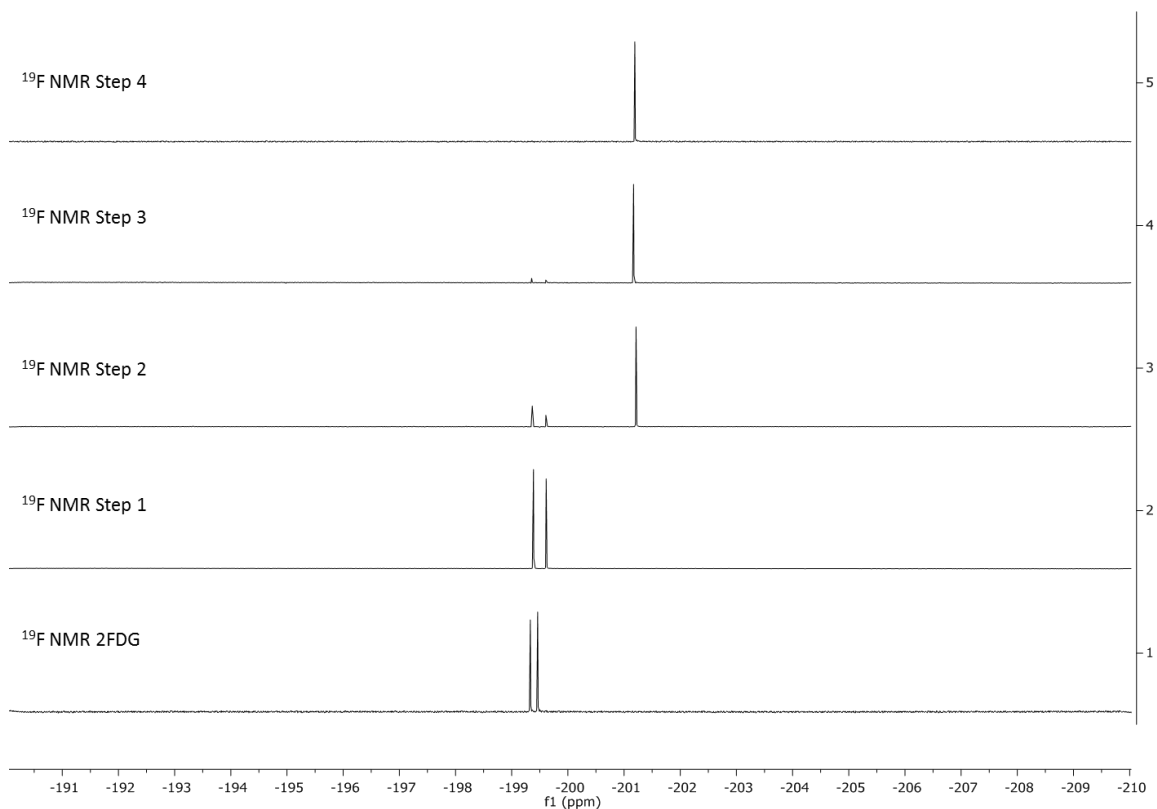


Figure S26. ^{19}F (Decoupled) NMR (470 MHz) spectra of crude mixture and purified product of ^{19}F -FDT.

Step 1 involves the conversion of **FDG** to **FDG6P**; Step 2 represents conversion of **FDG6P** to **FDT6P**; Step 3 shows the conversion of **FDT6P** to ^{19}F -FDT. Step 4 is for the purified final batch of ^{19}F -FDT. D_2O was used as solvent. For all crude reactions, 20 μL sample was diluted in 500 μL of D_2O ; whereas for the purified sample, 2.3 mg of ^{19}F -FDT was dissolved in 500 μL of D_2O .

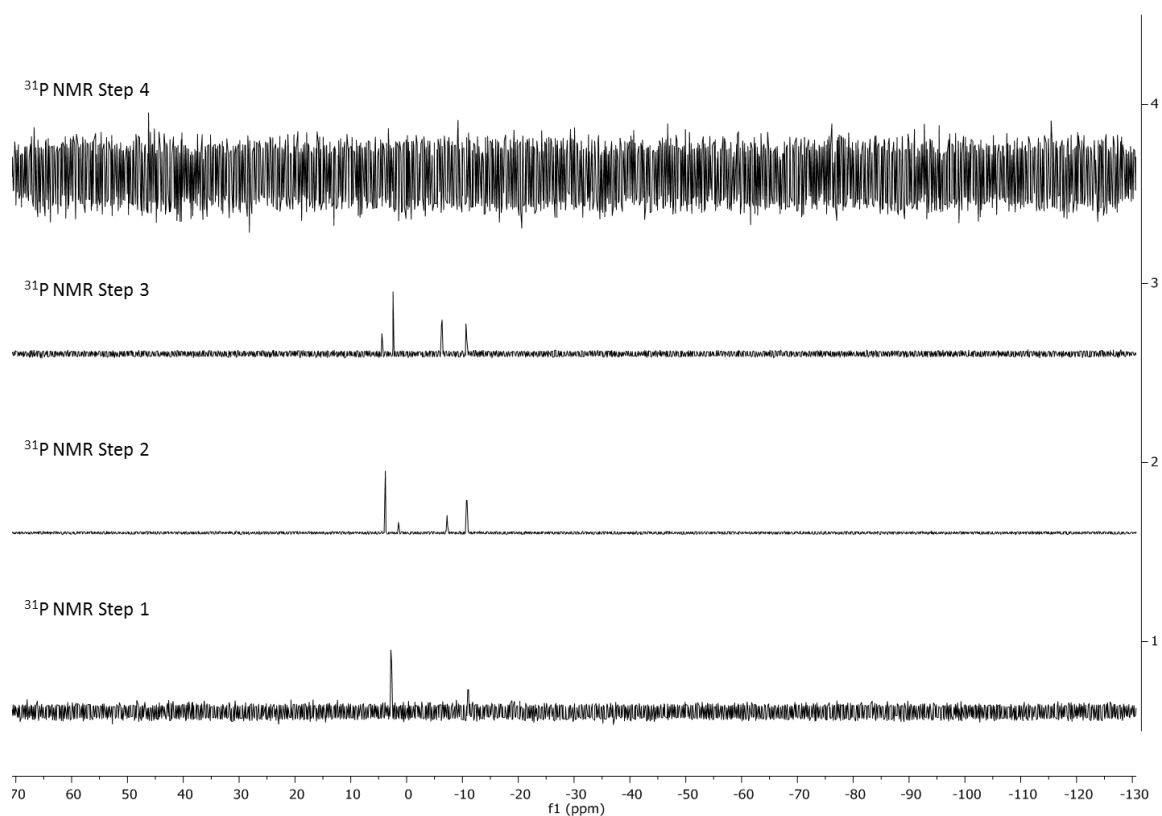


Figure S27. ^{31}P -NMR (202 MHz) spectra of crude mixture and purified batch of ^{19}F -FDT.

Step 1 involves the conversion of **FDG** to **FDG6P**; Step 2 represents conversion of **FDG6P** to **FDT6P**; Step 3 shows the conversion of **FDT6P** to ^{19}F -FDT. Step 4 is for the purified final batch of ^{19}F -FDT. D_2O was used as solvent. For all crude reactions, 20 μL sample diluted in 500 μL of D_2O ; whereas for the purified sample, 2.3 mg of ^{19}F -FDT was dissolved in 500 μL in D_2O .

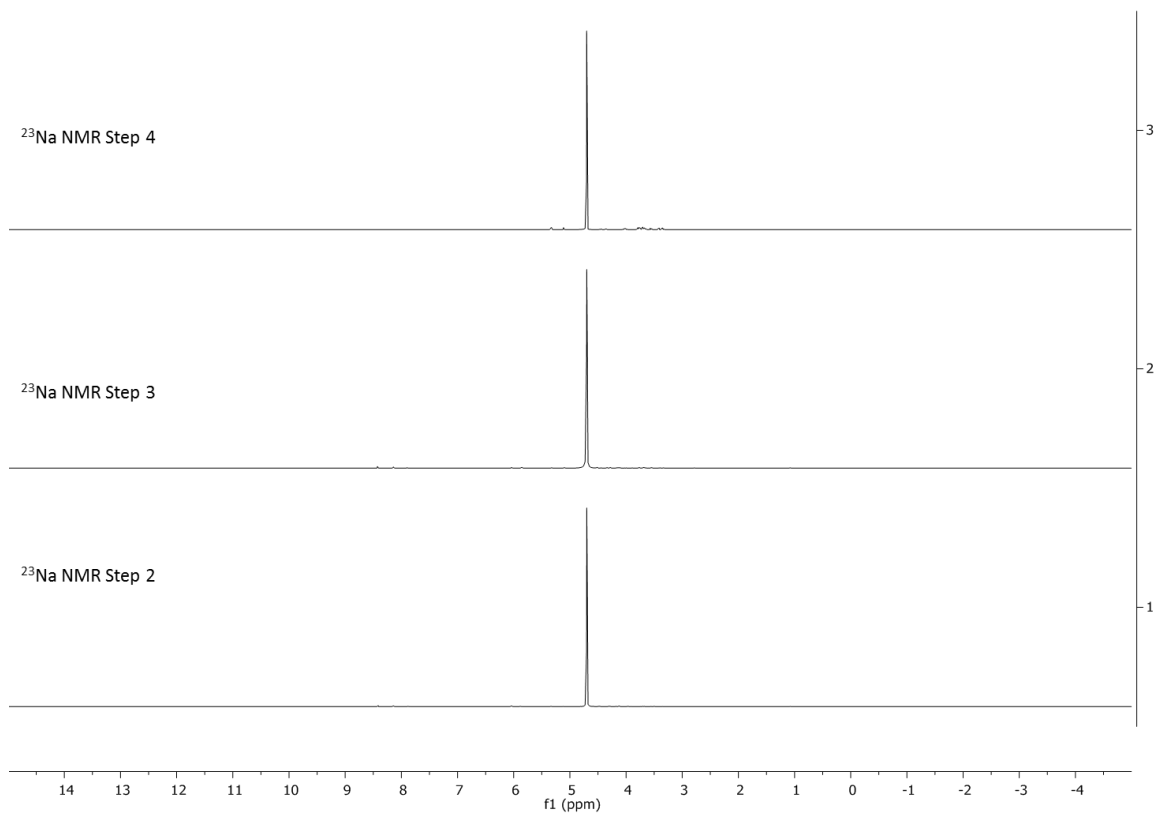


Figure S28. ^{23}Na -NMR (132 MHz) Spectra of FDT6P & [^{19}F]-FDT Crude and [^{19}F]-FDT purified batch.

Step 2 is from **FDT6P** crude mixture; Step 3 is from [^{19}F]-FDT crude mixture; Step 4 is from the purified product of [^{19}F]-FDT. All samples were analyzed in D_2O .

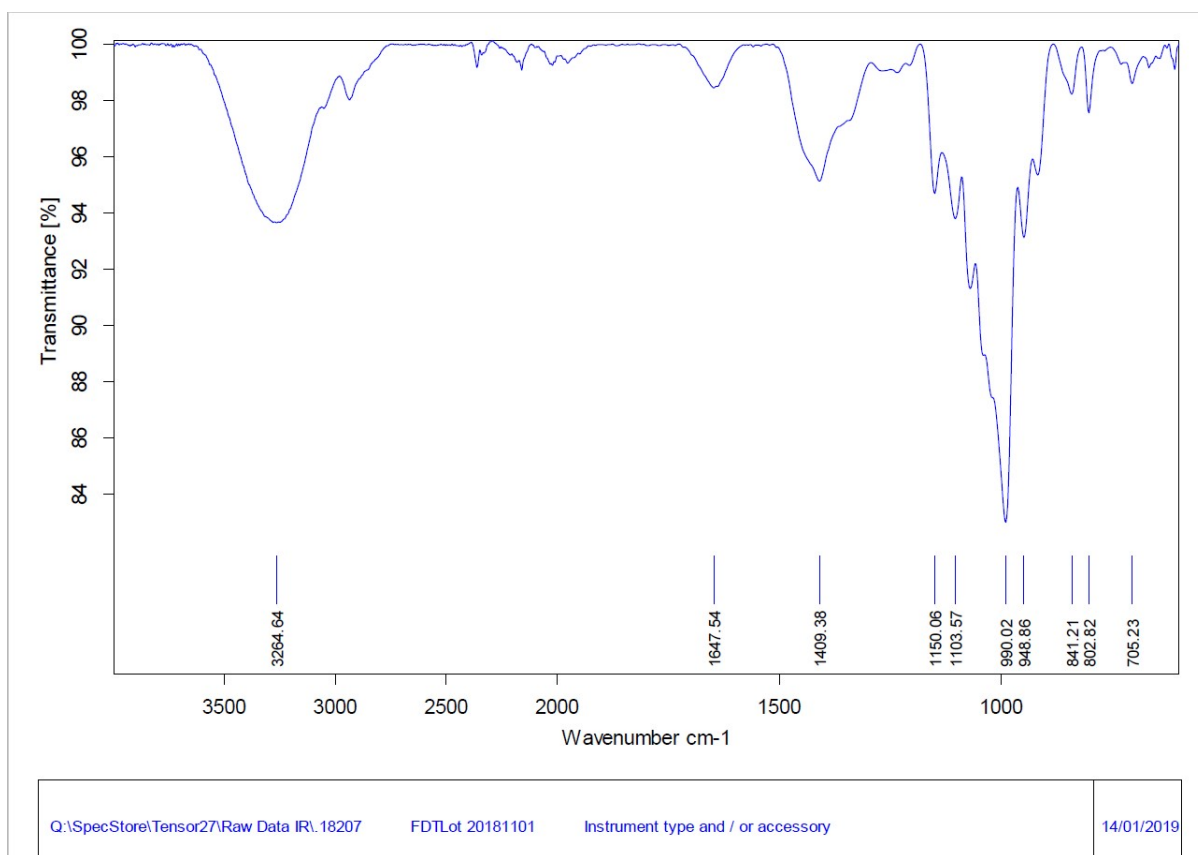


Figure S29. IR Spectrum of [¹⁹F]-FDT.

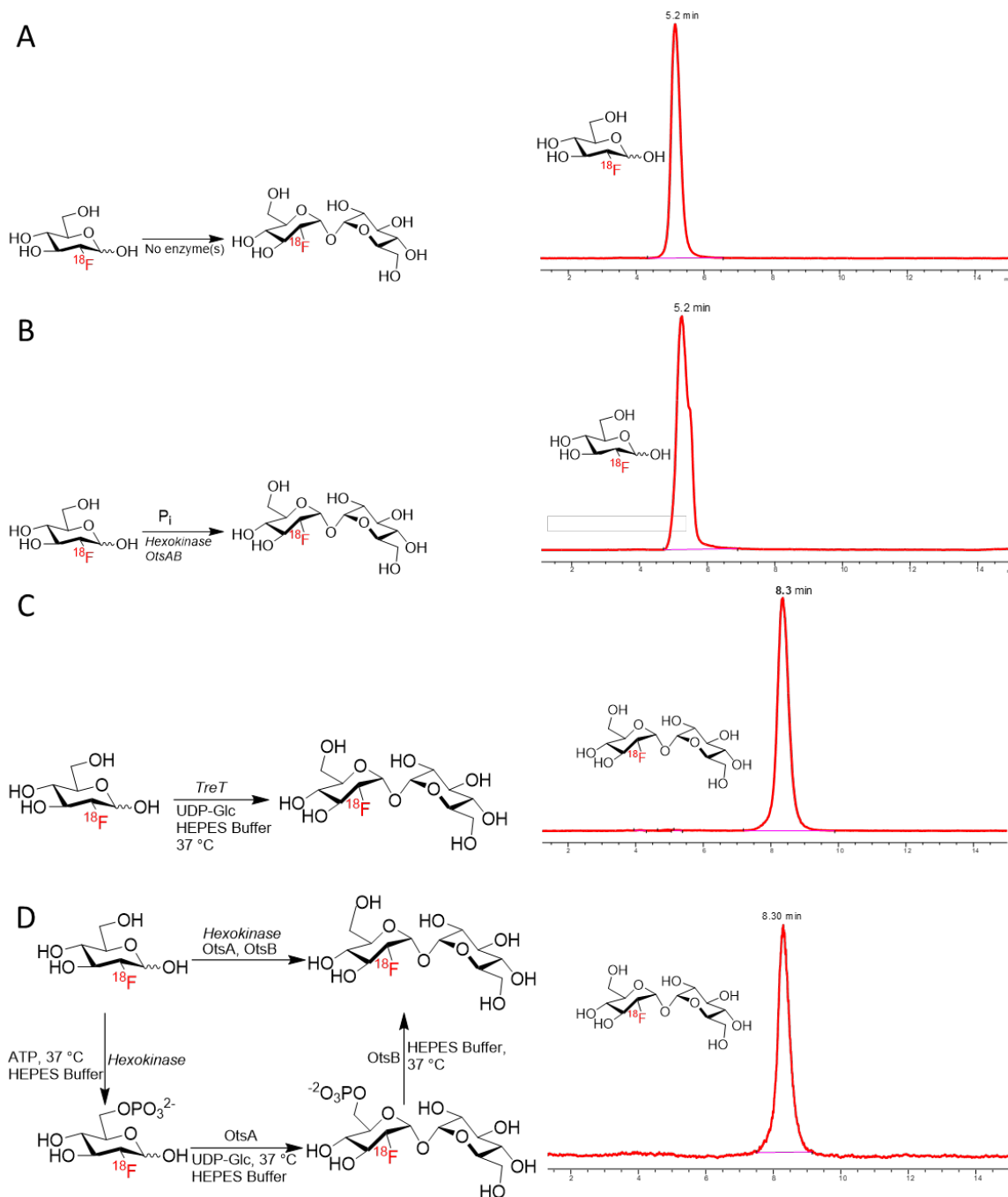


Figure S30. Enzymatic radiosynthesis of [¹⁸F]FDT.

A) Schematic overview and radio-HPLC chromatogram of control reaction, no enzyme was added in the reaction mixture. **B)** Schematic overview and chromatogram of attempted radiosynthesis of [¹⁸F]FDT using 2-enzyme-2-step one-pot synthesis method which failed to yield any product and only [¹⁸F]FDG observed after 2 h reaction. **C)** Schematic and radio-

HPLC chromatogram of [^{18}F]FDT synthesized from TreT enzyme; full conversion to the product observed after 60 min of reaction. **D**). Schematic overview of [^{18}F]FDT synthesised from hexokinase, OtsA and OtsB enzyme and radio-HPLC chromatogram; full conversion to the product observed after 60 min of reaction.

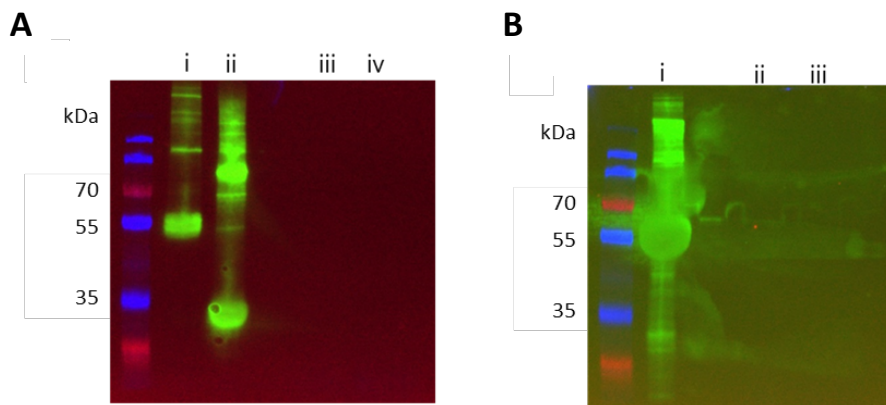


Figure S31. Western Blot Analyses of $[^{18}\text{F}]$ FDT Samples.

(A) Detection of OtsA and OtsB, using anti-His antibody. Lanes: (i) OtsA enzyme; (ii) OtsB enzyme; (iii) dilute $[^{18}\text{F}]$ FDT sample; (iv) concentrated $[^{18}\text{F}]$ FDT sample (B). Detection of hexokinase in $[^{18}\text{F}]$ FDT sample using anti-hexokinase antibody. Lanes: (i) hexokinase enzyme only; (ii) dilute $[^{18}\text{F}]$ FDT sample; (iii) concentrated $[^{18}\text{F}]$ FDT sample. No enzymes were detected in the $[^{18}\text{F}]$ FDT samples.

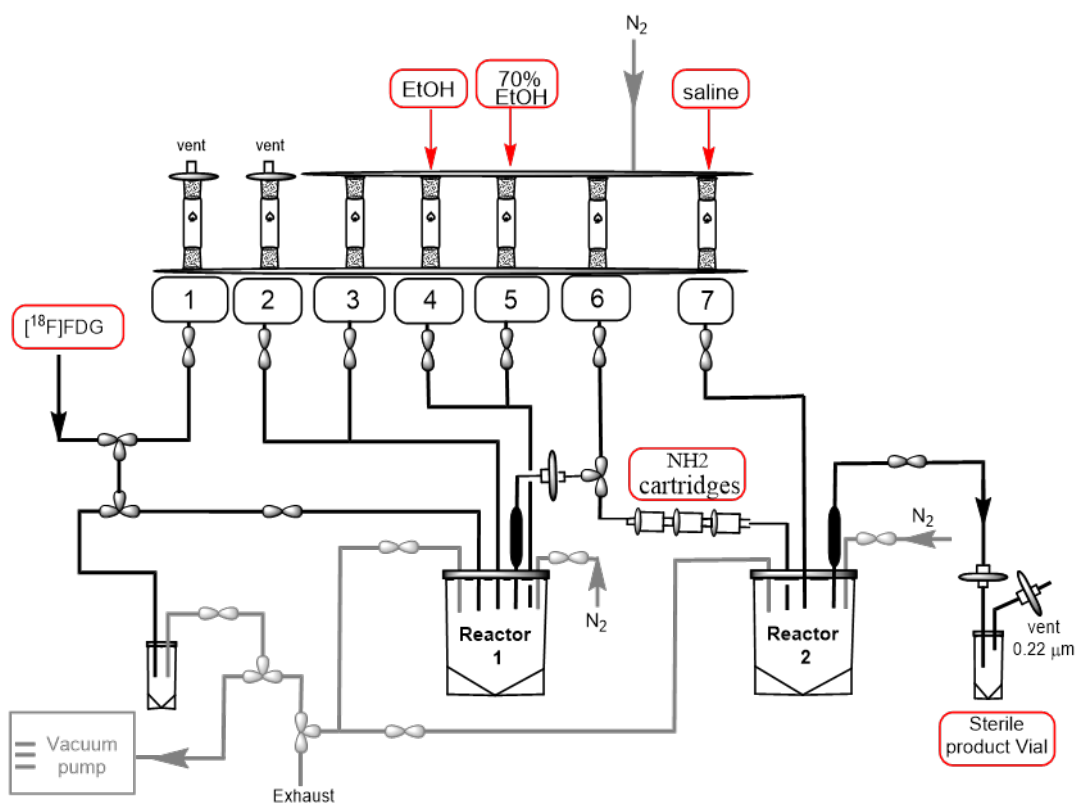


Figure S32. Schematic Diagram of Automated $[^{18}\text{F}]\text{FDT}$ Synthesis on GE-FXN pro module

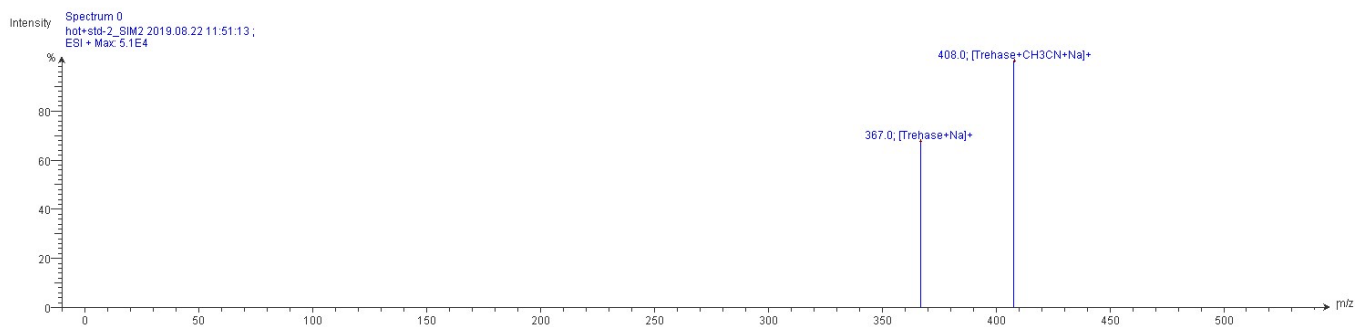
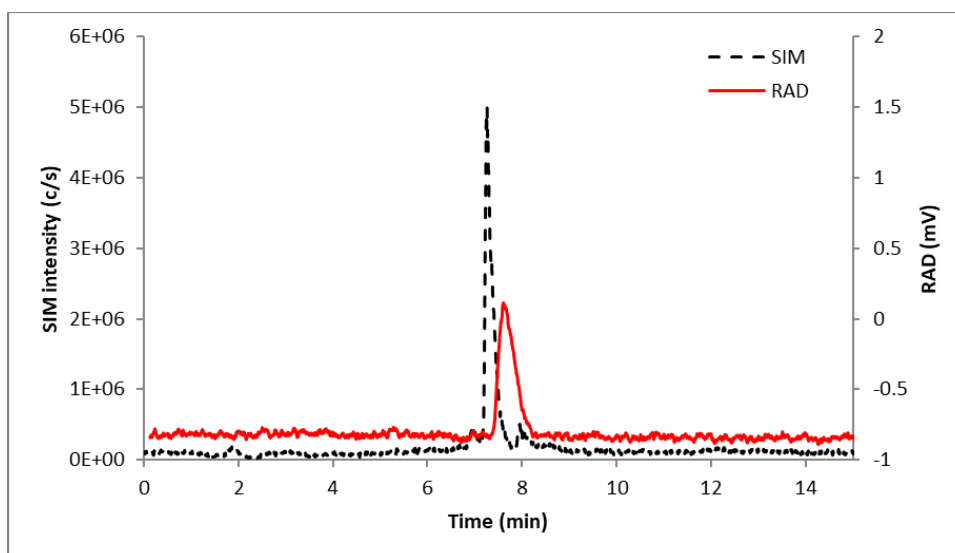


Figure S33. LC-MS analysis of [^{18}F]FDT coinjected with non-radioactive standard [^{19}F]FDT.

Top: in-line radiodetector (solid red line); SIM intensity of m/z 367 (black dotted line).

Bottom: corresponding ESI+ mass spectrum. Chromatographic Conditions: separations were carried out with gradient elution on an Waters XBridge Amide column, 130\AA , $3.5\ \mu\text{m}$, $4.6\ \text{mm} \times 150\ \text{mm}$. The mobile phase was composed of water (phase A) and acetonitrile (phase B) each containing 0.1 % ammonium hydroxide. The analytes were eluted from the column by a linear gradient which started at 90% phase B, then decreased to 50% phase B within 8 min. The total run time was 10 min. The flow rate was set at $1.0\ \text{mL}/\text{min}$. The column oven was kept at $40\ ^\circ\text{C}$ throughout the analysis. The injection volume was $20\ \mu\text{L}$.

Mass Spectrometric conditions: Mass spectrometric data were acquired in positive ion mode with the following ESI-MS parameters: Capillary Temperature 250 °C, Capillary Voltage 120 V, Source Voltage Offset 25V, Source Voltage Span 50V, Source Gas Temperature 200 °C, ESI Voltage 3,500V. Nitrogen was used as a desolvation gas. The analysis was done using scan/single ion monitoring (SIM) switching mode. The parameters for the scan mode was set as follows: scan range 101500 m/z, scan time 376 ms, scan speed 3963 m/z/sec. In SIM mode dwell time was set at 50 ms for all analytes. Non radioactive standard trehalose, FDT (m/z calculated mass for C₁₂H₂₁FO₁₀Na 367.10, found 408 [M+Na+ACN], 367 [M+Na]) at retention time 7.25 min was co-injected with radiolabeled product was used to confirm the identity of the analyte in the sample.

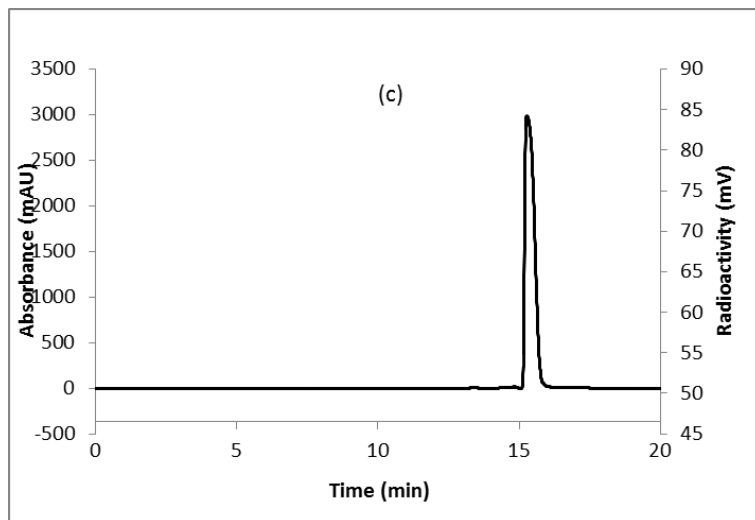
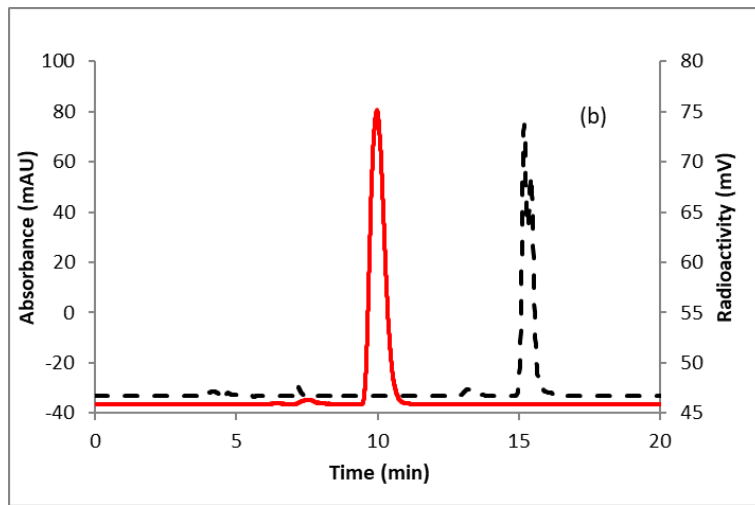
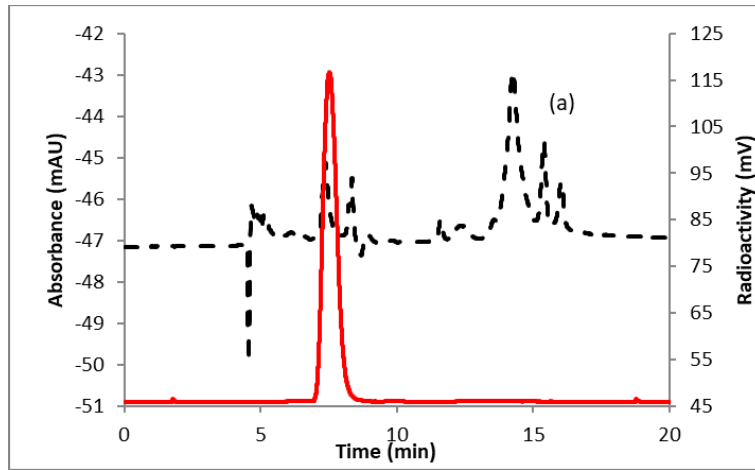


Figure S34. HPLC Analyses of (A) [¹⁸F]FDG; (B) [¹⁸F]FDT; (C) UDP-glucose.

4.6 x 250 mm, 2.7 μm AdvanceBio Glycan Mapping Column; solvent A = 50 mM ammonium formate, pH 4.5, solvent B = acetonitrile; Flow rate = 0.5 mL/min; gradient 0-15 min 68-62% B; 15-20 min 62-68% B. Retention. Red line, in-line radiodetection; black line, UV detection at 254 nm. The UV peak at ~15 min is for the UDP-glucose, confirmed by comparing the HPLC retention time of separately injected pure UDP glucose (A,C) and mass spec analysis of the decayed solution of [¹⁸F]FDT (B).

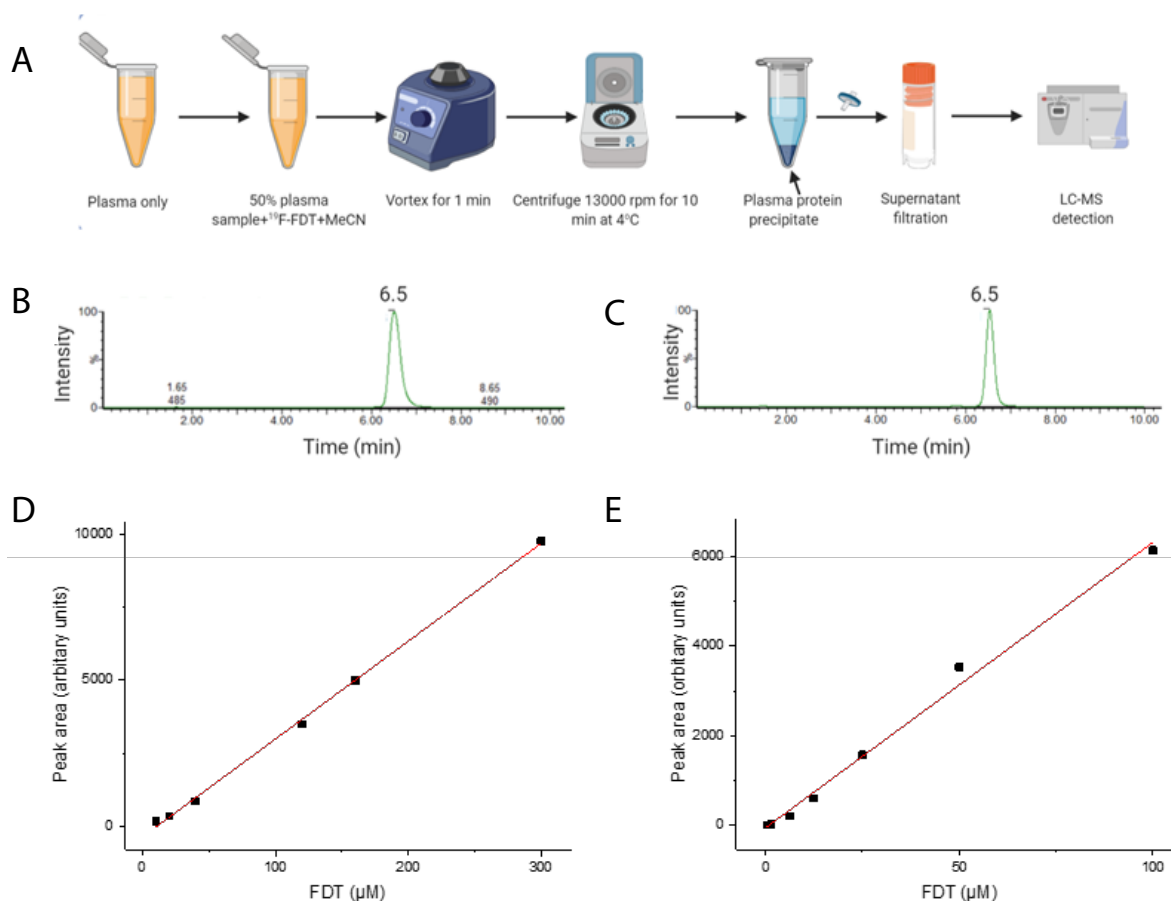


Figure S35. Detection of [¹⁹F]FDT for Assessment of Stability in Human Plasma

A) Experimental set-up of the sample preparation. Commercially available human plasma samples were spiked with known concentrations of [¹⁹F] FDT, plasma proteins were then precipitated by adding up to 50% of MeCN solution and after centrifugation the samples were analysed by LC-MS. Panel A created with [BioRender.com](https://www.biorender.com), released under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International license. **B)** [¹⁹F]FDT standard sample analysed by LC-MS method 2 with the retention time of 6.5 min. **C)** LC-MS analysis of plasma sample spiked with known amount of FDT showing the similar retention time to that of the standard sample with no immediate degradation or sequestration, allowing efficient detection. **D)** Calibration curve of plasma samples when spiked with FDT concentrations from 10 μM – 300 μM with $r^2 = 0.998$ and **E)** Further calibration curve showing instrument sensitivity down to > 390 nM for detection of FDT ($r^2 = 0.998$).

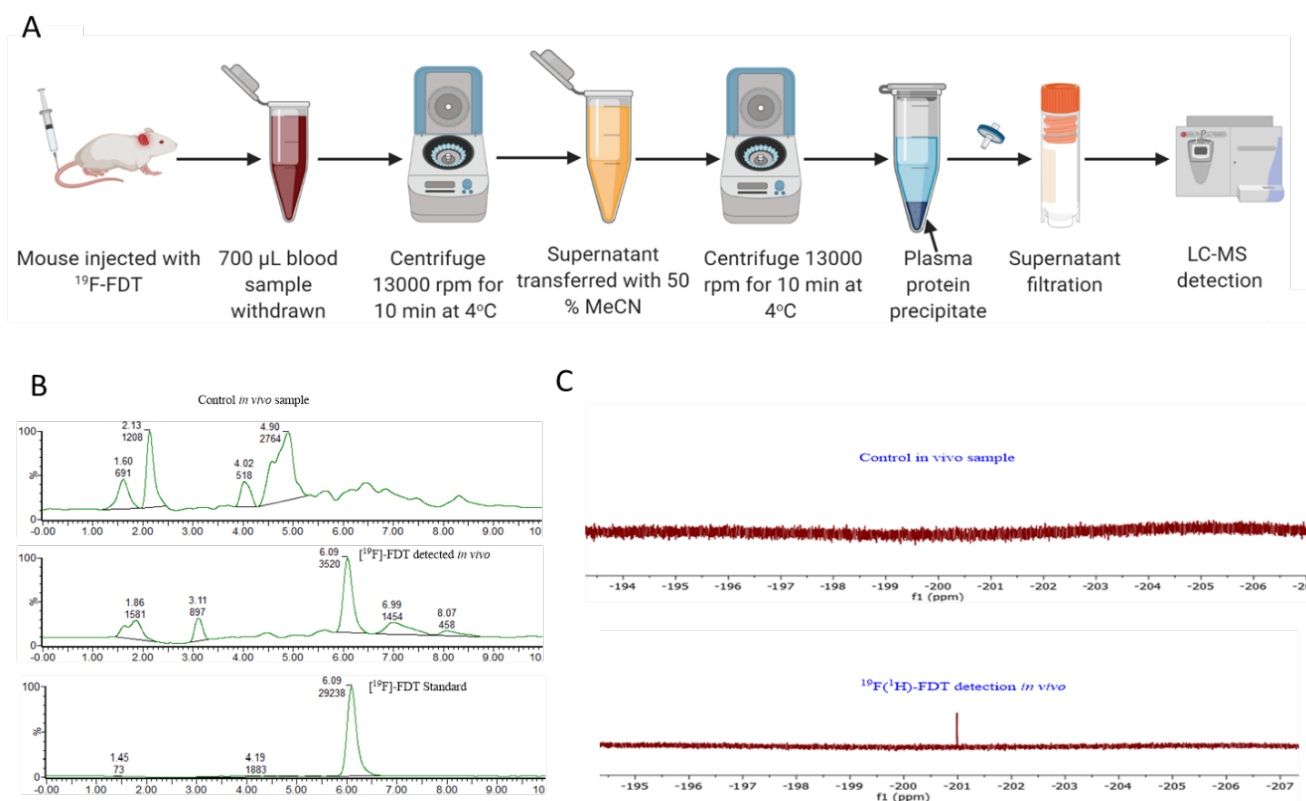


Figure S36. Method for Assessment of [^{19}F]FDT Stability *In vivo*.

A). Experimental set-up for the *in vivo* detection. Panel A created with [BioRender.com](https://www.biorender.com/), released under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International license. **B).** From top to bottom; control *in vivo* plasma sample; middle [^{19}F]FDT detection in plasma *sample in vivo* when 500 μM of the [^{19}F]FDT solution injected in mouse and sample taken 5 min post injection; [^{19}F]FDT standard sample; **C).** From top to bottom, control *in vivo* sample and bottom [^{19}F]FDT detection *in vivo* by ^{19}F NMR spectroscopy. See also Figure S37 and Figure S3G for time course metabolism data over an extended period for [^{19}F]FDT in marmosets/mice and [^{18}F]FDT in rabbits, respectively.

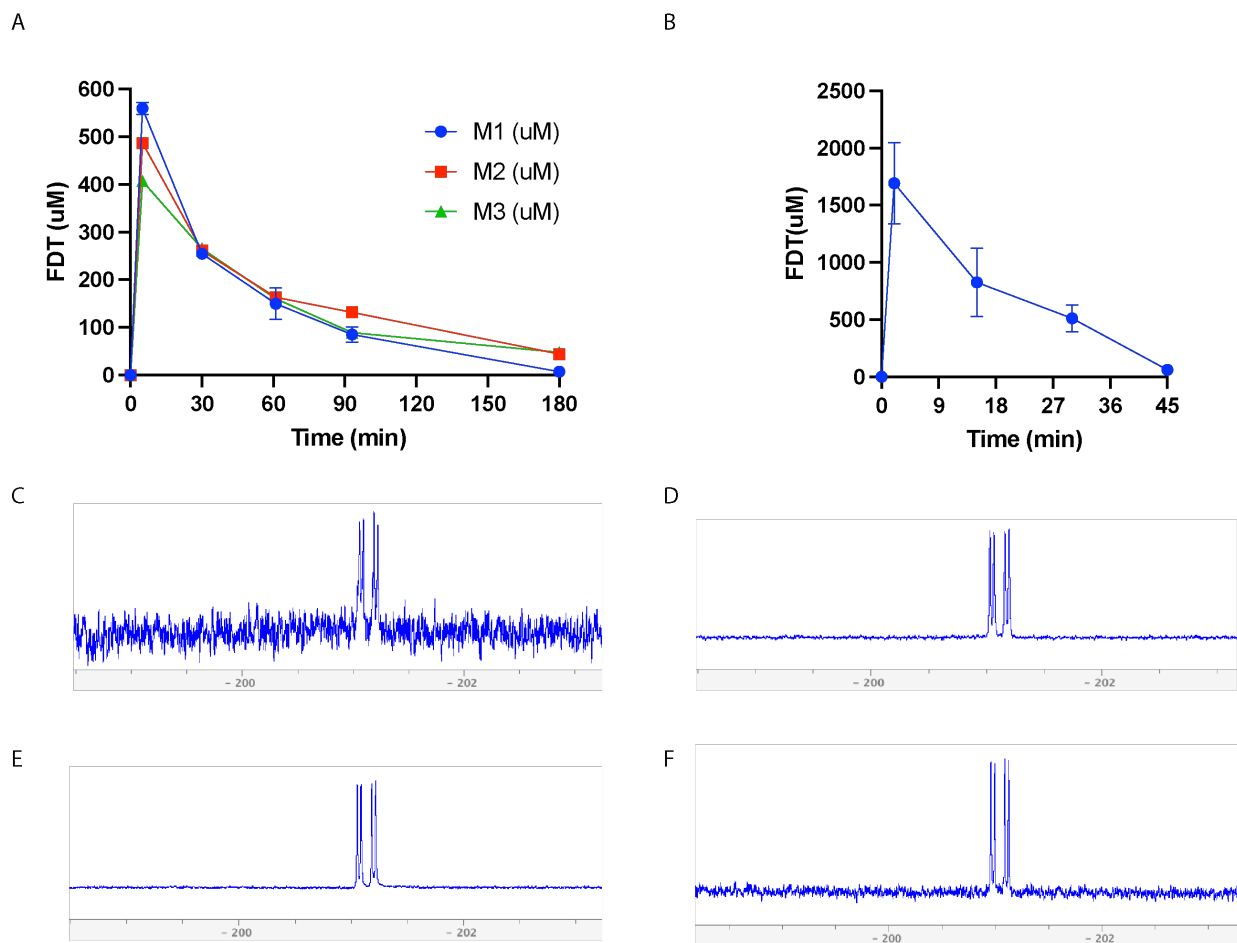


Figure S37. [¹⁹F]FDT Clearance and In Vivo Stability in Non-human Primates and Mice

A, B) 20 mg [¹⁹F]FDT was injected into marmosets (**A**, n=3 biological replicates, each measured in duplicate) and 5 mg FDT injected into mice (**B**, n=5 biological replicates, each measured in triplicate). FDT samples were analyzed using the method shown in Figure S36 in SIM mode. In both animal species the clearance of the compound showed normal behavior as shown in the time activity curve for marmosets (**A**) and mice (**B**). Under the limits of detection of these methods no degradation to [¹⁹F]FDG was detected, consistent with the only low levels of metabolism seen with [¹⁸F]FDT in rabbits (see Figure S3G). **C-F**) We further analyzed the potential degradation of FDT into FDG by ¹⁹F NMR spectroscopy in marmoset plasma (**C**) sample from 5 min time point, mouse plasma (**D**) sample from 30 min time point and mouse urine samples from 30 min (**E**) and 45 min (**F**) time points, respectively. In all these samples only [¹⁹F]FDT was observed (δ -201.2). The data points are average values from three replicates with error bars \pm SD.

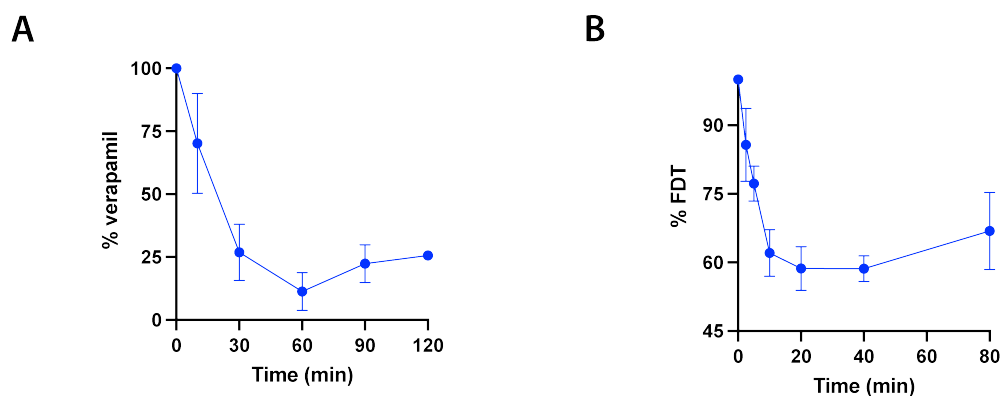


Figure S38. [¹⁹F]FDT Stability in Human Liver Microsomes.

Human liver microsomal stability assays were conducted *in vitro* (**A**, **B**) (n = 2, biological replicates), using verapamil as a comparison (**A**); for verapamil ~ 19% drug remained after 30 min. Comparatively, in the case of [¹⁹F]FDT, > 50% of compound remained after 80 min (**B**). The data points are average values from 2 replicates with error bars ± SD.

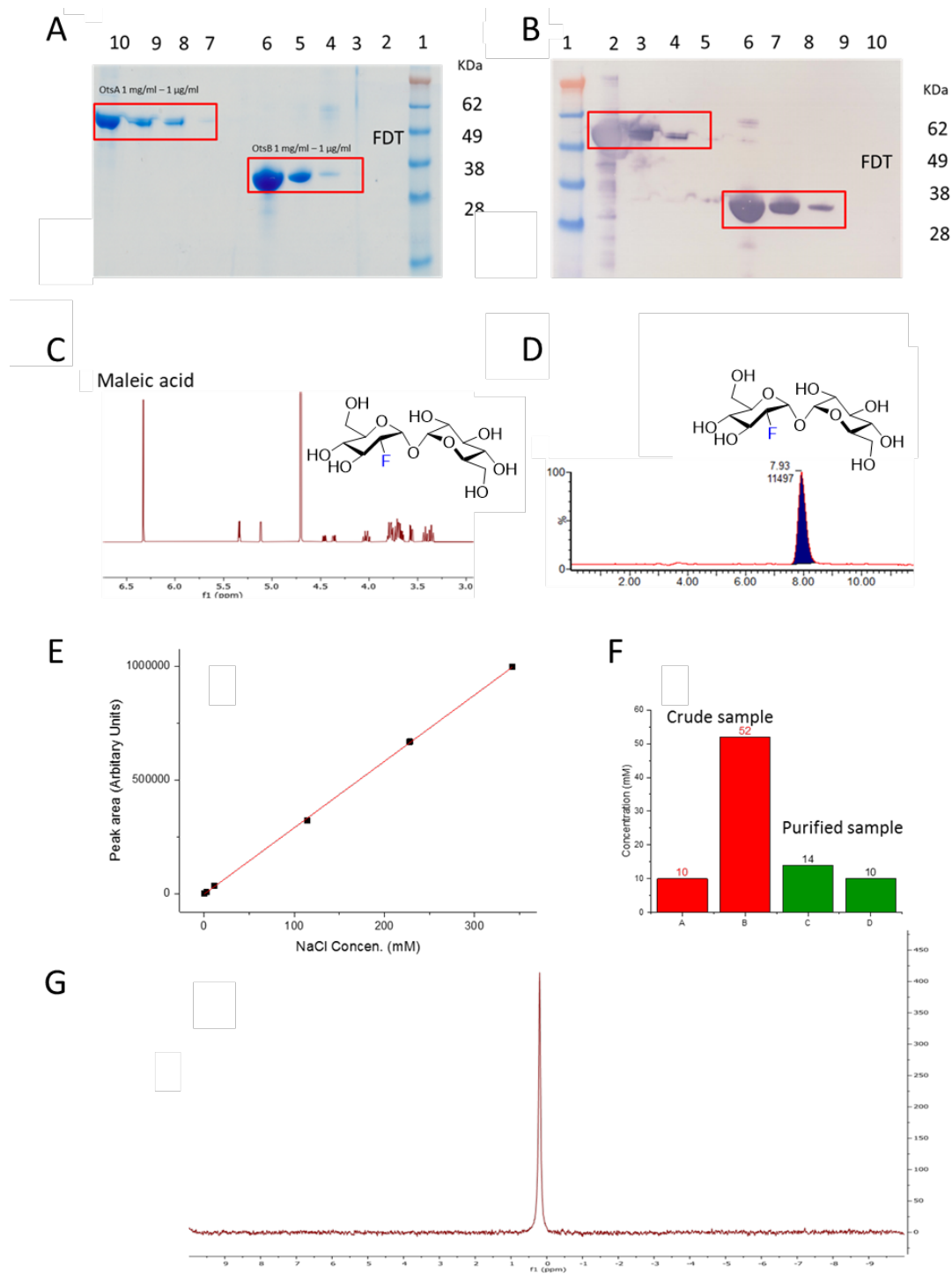


Figure S39. FDT Batch Quantification and Purity Testing.

(A) SDS-PAGE analysis for detection of and enzyme leaching in the final FDT batch. Lanes: OtsA standards (lane 7-10) and OtsB standards (lane 3-6) with 1/10 serial dilutions loaded onto the SDS-PAGE. 25 mg/mL FDT from purified batch also loaded on the gel (lane 2). No

enzyme was detected in the FDT sample. **(B)** Western blotting visualisation for the detection of enzyme leaching in the final FDT batch. Lanes: OtsA standard (lane 2-5) and OtsB standards (lane 6-9) enzymes with 1/10 serial dilutions from 1mg/mL to 1 µg/mL loaded onto the SDS-PAGE. 25 mg/mL FDT (lane 10) from purified batch also loaded. Visualisation was done with an anti-His monoclonal antibody. No protein was detected in the FDT sample even at this high concentration. **(C)**. Processed quantitative ¹H-NMR spectrum of analyte FDT. Maleic acid as an internal standard. **(D)** LC-MS Chromatogram of FDT. Retention times above; integral below for each noted peak. Peak at 7.93 min is FDT with major ESI⁻ m/z of 343. **(E)** Standard calibration curve for NaCl using ²³Na-NMR; **F)** Concentrations in FDT samples (determined by combined ¹⁹F-NMR and ²³Na-NMR) in the crude reaction mixture (red) and purified (green). Column A is the concentration of FDT in the crude reaction mixture determined by ¹⁹F-NMR; column B shows the quantified value of NaCl as determined by ²³Na-NMR. Columns C & D are the corresponding measurements from the purified sample of FDT. All are calculated from samples dissolved in 0.5 mL D₂O. Total integrals from the peak area were used for calculation using intercept and slope from the calibration curve. **(G)** Example ²³Na-NMR spectrum for quantitative yield calculation of NaCl in samples.

Supplementary Tables

Table S1. Endotoxin test for the one-pot reaction mixture containing OtsA and OtsB from *E. coli* at each purification step.

Endotoxin assay was carried out using Endosafe™-PTS detection instrument (Charles River). Detection cartridges were 0.01 – 1.0 endotoxin unit (EU)/mL and 0.05 – 5.0 EU/mL. Protein concentration at each purification step was also carried out at the same time using BCA method (Thermo Scientific). The measured values for endotoxins were not averaged but presented as the range of measured concentrations while protein concentrations were averaged

Samples	Protein (mg/mL)	Endotoxins (EU/mL)
Reaction mixture	> 3	> 5,000
After 1 st SAX SPE	0.43	200 – 392
After 2 nd SAX SPE	Background level	0.05 – 5
After 3 rd SAX SPE	Background level	0.01 – 0.051
After HPLC	Background level	< 0.01

Table S2. Effect of amount of [¹⁸F]FDG on radiochemical yield

Type of Synthesis	Entry	[¹⁸ F]FDG mCi	[¹⁸ F]FDG Volume mL	Total Volume mL	% Yield, non-decay corrected
Automated	1	40	0.8	1.1	26.3 ± 5.8 (n = 2)
	2	60	1.0	1.3	26.3 ± 5.8 (n = 2)
	3	100	1.8	2.1	41.0 ± 4.2 (n=3)

Table S3. LC-MS Analysis Gradient for LCT Classic MS

Time (min)	% Solvent A	% Solvent B	Flow rate (mL/min)
0.0	95	5	0.4
1.0	95	5	0.4
7.0	5	95	0.4
11.0	5	95	0.4
12.0	95	5	0.4
17.0	95	5	0.4

Table S4. LC-MS Analysis Gradient for Waters Xevo G2-QS QToF MS

Time (min)	% Solvent A	% Solvent B	Flow rate (mL/min)
0.0	95	5	0.4
1.0	95	5	0.4
7.0	5	95	0.4
8.0	5	95	0.4
8.1	95	5	0.4
10.0	95	5	0.4

Table S5: Summary of different HPLC conditions for enzymatic reaction analysis

Method	Mobile Phase	Stationary	Gradient	Flow rate	Run	UV
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		Phase	(mL/min)	time	detection	
				(min)	(λ)	
1	A. H ₂ O	waters	5-100% B	1	30	262
	B. Ammonium formate 300 mM	spherisorb 5 μm SAX 4.6 x 250 nm	linear gradient			
2	A. H ₂ O	waters	5-100% B	1	30	262
	B. H ₂ KPO ₄ 100 mM	spherisorb 5 μm SAX 4.6 x 250 nm	linear gradient			
3	A. H ₂ O	Phenomenex	0-100%	60	0.6	65
	B. H ₂ KPO ₄ 1M	Luna NH ₂ 4.6 x 250 nm	linear gradient			
			over min, 61-65 min, 100%			
			A			

Table S6: Reagents Details for Enzyme Comparison Reaction

Enzyme	FDG (mM)	UDP-Glc (mM)	MgCl ₂ (mM)	Enzyme (μM)	HEPES Buffer (mM)
TreT <i>P. horikoshii</i>	10	20	10	1.2	50 mM, pH 7.5
TreT <i>T. tenax</i>	10	20	10	1.2	50 mM, pH 7.5

Table S7. Testing ATP concentrations for the conversion of FDG to FDG6P

Entry	FDG (mM)	ATP (mM)	MgCl ₂ (mM)	Enzyme (mg)	HEPES Buffer (mM)
1	5.55	2.75	10	5	50 mM, pH 7.5
2	5.55	5.50	10	5	50 mM, pH 7.5
3	5.55	8.25	10	5	50 mM, pH 7.5
4	5.55	16.5	10	5	50 mM, pH 7.5

Table S8: Reagent Amounts Used in the Small scale Synthesis of FDT Using a 3-enzyme-one-pot System

Entry	FDG (mmol)	ATP (mmol)	MgCl ₂ (mmol)	KCl (mmol)	UDP-Glc (mmol)	hexokinase (mg)	OtsA (mg)	OtsB (mg)	HEPES (50 mM)	Reaction volume (mL)
1	0.1380	0.7259	5	100	20	1	1	0.1	Diluent	1
2	0.2760	0.7259	5	100	20	1	1	0.1	Diluent	1
3	0.4140	0.7259	5	100	20	1	1	0.1	Diluent	1
4	0.5520	0.7259	5	100	20	1	1	0.1	Diluent	1
5	1.1040	7.0780	5	100	20	1	1	0.1	Diluent	1
6	2.2082	7.0780	5	100	20	1	1	0.1	Diluent	1
7	4.4164	7.0780	5	100	20	1	1	0.1	Diluent	1
8	5.5205	7.0780	5	100	20	1	1	0.1	Diluent	1

Table S9. LC-MS methods employed in the detection of FDT

Method no.	Mobile phase	Flow rate (mL/min)	Run time (min)	Temperature (°C)	FDT rt (min)
1	30/70 H ₂ O/MeCN	0.8	20	25	10.5
2	35/65 H ₂ O/MeCN	0.3	12	25	8.7
3	35/65 H ₂ O/MeCN	0.4	12	25	6.5
4	35/65 H ₂ O/MeCN	0.4	12	60	6.1

Table S10: Experimental Design for Toxicity Studies in Rats

Group	Treatment	Dose Level (mg/kg/day)	No. Doses	Total Dose (mg/kg)	Main groups animal	Recovery Group animals
1	Vehicle	0	7	0	10M/10F	5M/10F
2	100 x human dose	1.32	7	9.24	10M/10F	5M/10F
3	100 x human dose	1.32	1	1.32	10M/10F	5M/10F
4	1000 x human dose	1.32	1	1.32	10M/10F	5M/10F

Table S11: Experimental Design for Toxicity Studies in Dogs

Group	Treatment	Dose Level (mg/kg/day)	No. Doses	Total Dose (mg/kg)	Main groups animal	Recovery Group animals
1	Vehicle	0	7	0	3M/3F	2M/2F
2	100 x human dose	0.4	7	2.8	3M/3F	2M/2F
3	100 x human dose	0.4	1	0.4	3M/3F	2M/2F
4	1000 x human dose	4	1	4	3M/3F	2M/2F

Supplementary References

- 1 Carillo, P. *et al.* A fluorometric assay for trehalose in the picomole range. *Plant Methods* **9**, 21 (2013). <https://doi.org:10.1186/1746-4811-9-21>
- 2 Guallar-Garrido, S., Luquin, M. & Julián, E. Analysis of the Lipid Composition of Mycobacteria by Thin Layer Chromatography. *JoVE*, e62368 (2021). <https://doi.org:doi:10.3791/62368>
- 3 Backus, K. M. *et al.* Uptake of unnatural trehalose analogs as a reporter for Mycobacterium tuberculosis. *Nature Chemical Biology* **7**, 228-235 (2011). <https://doi.org:10.1038/nchembio.539>