

## Supporting Information

For

### One-step synthesis of $\alpha$ -Gal epitope and globotriose derivatives by an engineered $\alpha$ -galactosidase

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## 1. Enzyme assay

The  $\alpha$ -D-galactosidase activity was assayed by adding 30  $\mu$ L of enzyme solution to 150  $\mu$ L of 2 mM *p*-nitrophenyl  $\alpha$ -D-galactopyranoside (*p*NPGal, Sigma) solution. The reaction was performed at 37 °C for 10 min, and then quenched by adding 1.05 mL of 0.2 M sodium borate buffer (pH 10.5). The amount of *p*-nitrophenol released was measured at 400 nm using a UV-Visible spectrometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1  $\mu$ M of *p*-nitrophenol per minute under the same condition.

## 2. Random mutagenesis of $\alpha$ -galactosidase (*Aga2*) from *Bifidobacterium breve* 203

The  $\alpha$ -galactosidase gene (*aga2*, GenBank accession number DQ267828) was subjected to random mutagenesis by error-prone PCR. Plasmid pET-22b-*aga2* constructed in our previous report was used as the template DNA. The forward and reverse primers were (5'-CTCTCATATGATGGCAATCATGGATTTCCACGGGAG-3') and (5'-ATATGGATCCGCTCTCACGCGCGTGGCGCTGAAC-3') (underlined are *Nde* I and *Bam*H I restriction sites). The PCR amplification was processed in a 50  $\mu$ L reaction containing template DNA (20 ng), forward and reverse primers (0.4  $\mu$ M each), 10 $\times$ buffer (5  $\mu$ L), *Taq* DNA polymerase (2.5 unit), a high concentration of MgCl<sub>2</sub> (7 mM) and a skewed ratio of dNTPs (0.2 mM dATP and dGTP; 1 mM dTTP and dCTP). The high concentration of MgCl<sub>2</sub> and skewed ratio of dNTPs were used to reduce the fidelity of the *Taq* polymerase and introduce random point mutations. PCR programs started with a heating step at 94 °C for 5 min, then 30 cycles at 94 °C for 35 s, 57 °C for 30 s, 72 °C for 2.5 min, and at last 72 °C for 10 min. PCR products were digested by the restriction enzymes and cloned back to pET-22b for C-His<sub>6</sub>-tagged protein. The resultant ligation mixture was transformed into chemical competent *Escherichia coli* XL 1-Blue cells (Takara). The transformants were spread on LB medium (10 g/L tryptone, 5 g/L yeast extract and 7.5 g/L NaCl) plate containing 10  $\mu$ g/mL 5-bromo-4-chloro-3-indolyl- $\alpha$ -D-galactopyranoside (*X- $\alpha$ -gal*) and 50  $\mu$ g/mL ampicillin. After growth at 37 °C overnight, about 4000 recombinant clones formed on plates and 335 blue clones were picked out and cultured for plasmid extraction. The recombinant plasmids were

transformed into chemical competent *E. coli* BL21 (DE3) cells for protein expression. After overnight induction, 5 mL culture of each recombinant was harvested and the cell pellet was suspended in 50  $\mu$ L of sodium acetate buffer (50 mM, pH 5.5), freeze-thawed three times, and then incubated with 0.2 M melibiose at 37  $^{\circ}$ C for 5 h to be screened for transglycosylation activity. The transglycosylation activity was finally detected by comparing the spot size of transglycosylation products on TLC. Two mutants RM70 and RM103 with improved transglycosylation activity were obtained. DNA sequencing performed by BioSune Company (China) revealed that three mutations G218S, D457A and H729R occurred in mutant RM70 and two mutations V564E and H573L were in mutant RM103.

### 3. Prediction of catalytic residues

The catalytic general acid/base and nucleophile residues of Aga2 were predicted by the multiple alignment of the amino acid sequences of Aga2 and other  $\alpha$ -galactosidases with determinate catalytic residues based on their crystal structures, and all of these selected  $\alpha$ -galactosidases were from the glycoside hydrolase family 36. The multiple alignment was performed by the ClustalW program (<http://www.ebi.ac.uk/clustalw/>). According to the result of multiple alignment, the catalytic general acid/base and nucleophile residues of Aga2 were estimated to D537 and D471, respectively.

<i>G. stearothermophilus</i> (AgaA)	ISNVLASAPITYV <b>K</b> WDMNRHMTEIGSSALPPERQRETAHRYMLGLYRVMD <b>E</b> ITSRFP <b>H</b> IL	522
<i>G. stearothermophilus</i> (AgaB)	ISNVLASAPITYV <b>K</b> WDMNRHMTEIGSSALPPERQRETAHRYMLGLYRVMD <b>E</b> MTSRFP <b>H</b> IL	522
<i>R. gnavus</i> E1	ICVVL <b>D</b> Q <b>G</b> KIDYV <b>K</b> WDMN <b>R</b> SMADVYAG <b>N</b> LS-----YDYVLGVYDFMERLCSRY <b>P</b> DL <b>L</b>	514
<i>L. acidophilus</i> NCFM	MVKILDSK <b>K</b> IDY <b>I</b> K <b>W</b> DMN <b>R</b> SLSDI <b>E</b> SDLPAD <b>Q</b> Q <b>E</b> AYHRYVLGY <b>D</b> LLN <b>K</b> LVTRY <b>P</b> D <b>I</b> L	526
<i>B. breve</i> 203	MDALVSEL <b>G</b> IDY <b>I</b> K <b>W</b> DH <b>N</b> KYVTEAVSP <b>R</b> T <b>G</b> ---RPA <b>V</b> H <b>G</b> QTLAVYR <b>I</b> FR <b>D</b> L <b>K</b> TA <b>H</b> PG <b>L</b> E	511
<i>G. stearothermophilus</i> (AgaA)	F <b>E</b> SCSGGGGRFD <b>P</b> G <b>M</b> LY <b>M</b> P <b>Q</b> T <b>W</b> T <b>S</b> D <b>N</b> TDA <b>V</b> S <b>R</b> L <b>K</b> I <b>Q</b> Y <b>G</b> T <b>S</b> L <b>V</b> P <b>I</b> S <b>A</b> M <b>G</b> A <b>H</b> V <b>S</b> A <b>V</b> P <b>N</b> H <b>Q</b>	582
<i>G. stearothermophilus</i> (AgaB)	F <b>E</b> SCSGGGGRFD <b>P</b> G <b>M</b> LY <b>M</b> P <b>Q</b> T <b>W</b> T <b>S</b> D <b>N</b> TDA <b>V</b> S <b>R</b> L <b>K</b> I <b>Q</b> Y <b>G</b> T <b>S</b> L <b>V</b> P <b>I</b> S <b>A</b> M <b>G</b> A <b>H</b> V <b>S</b> A <b>V</b> P <b>N</b> H <b>Q</b>	582
<i>R. gnavus</i> E1	LE <b>G</b> CSGGGG <b>R</b> FD <b>A</b> G <b>M</b> LY <b>S</b> P <b>Q</b> I <b>W</b> C <b>S</b> D <b>N</b> TDA <b>I</b> N <b>R</b> T <b>R</b> I <b>Q</b> Y <b>G</b> T <b>S</b> FF <b>Y</b> P <b>V</b> S <b>A</b> M <b>G</b> A <b>H</b> V <b>S</b> A <b>V</b> P <b>N</b> H <b>Q</b>	574
<i>L. acidophilus</i> NCFM	F <b>E</b> GC <b>S</b> GGGG <b>R</b> FD <b>V</b> G <b>Q</b> A <b>Y</b> T <b>P</b> Q <b>I</b> W <b>A</b> S <b>D</b> N <b>T</b> DA <b>I</b> ER <b>L</b> K <b>I</b> Q <b>Y</b> G <b>T</b> S <b>L</b> V <b>P</b> Q <b>S</b> M <b>M</b> T <b>S</b> H <b>V</b> S <b>V</b> S <b>P</b> N <b>E</b> Q	586
<i>B. breve</i> 203	I <b>E</b> SC <b>S</b> GGGG <b>R</b> VD <b>L</b> A <b>I</b> LS <b>L</b> AD <b>R</b> I <b>W</b> A <b>S</b> D <b>C</b> VD <b>P</b> VER <b>A</b> D <b>I</b> Q <b>R</b> Y <b>T</b> S <b>L</b> LV <b>P</b> PE <b>M</b> I <b>G</b> E <b>H</b> V <b>G</b> A <b>S</b> PA <b>H</b> S	571

**Figure S1.** Multiple alignment of the general acid/base and nucleophile residues of  $\alpha$ -galactosidases from *Bifidobacterium breve* 203 (Aga2, GenBank accession no. ABB76662), *Lactobacillus acidophilus* NCFM

(AAO21867.1), *Ruminococcus gnavus* E1 (CCA61959.1), and *Geobacillus stearothermophilus* (AgaA, AAG49420.1; AgaB, AAG49421.1). ▲, nucleophile residue; ●, general acid/base residue.

#### 4. Site-directed mutagenesis of Aga2

The targeted mutations were generated using the Easy Mutagenesis System kit (TransGen) following the manufacturer's directions. Plasmid pET-22b-*aga2* was used as the template, and *LA Taq* polymerase (TaKaRa) was used for PCR. The primers for amplification and mutagenesis were custom-synthesized and shown in Table S1. The resulting PCR products were transformed into chemical competent *E. coli* DH5 $\alpha$  cells. Selected clones were grown for minipreps, and positive clones were verified by DNA sequencing and were transformed into chemical competent *E. coli* BL21 (DE3) cells for protein expression.

**Table S1.** Primers used for site-directed mutagenesis.

Primers	Sequences (5'→3')
G218S-F	CTGCTGCTGAACGTG <u>TCC</u> GCCCCGGCTTC
G218S-R	CCACGTTTCAGCAGCAGCGACGAATCG
D457A-F	GTACGGCTGCATG <u>GCA</u> GCGCTTGTCAGCGA
D457A-R	TCGCTGACAAGCGCATCCATGCAGCCGTAC
V564E-F	ATGATCGGCGAACATG <u>AA</u> GGCGCGAGCCCCGC
V564H-F	ATGATCGGCGAACAT <u>CAC</u> GGCGCGAGCCCCGC
V564R-F	ATGATCGGCGAACAT <u>CGC</u> GGCGCGAGCCCCGC
V564N-F	ATGATCGGCGAACATA <u>AAC</u> GGCGCGAGCCCCGC
V564D-F	ATGATCGGCGAACATG <u>AC</u> GGCGCGAGCCCCGC
V564S-F	ATGATCGGCGAACATT <u>TCC</u> GGCGCGAGCCCCGC
V564Y-F	ATGATCGGCGAACATT <u>TAC</u> GGCGCGAGCCCCGC
V564W-F	ATGATCGGCGAACATT <u>TGGG</u> GCGCGAGCCCCGC
V564I-F	ATGATCGGCGAACATA <u>ATC</u> GGCGCGAGCCCCGC
V564M-F	ATGATCGGCGAACATA <u>ATG</u> GGCGCGAGCCCCGC
V564-R <sup>a</sup>	ATGTTCCGCGATCATCTCCGGCGGC

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H573L-F	CCCGCGCATTCCACCCTCCGTGCGACGAGCCA
H573R-F	CCCGCGCATTCCACCCGCCGTGCGACGAGCCA
H573N-F	CCCGCGCATTCCACCAACCCTGCGACGAGCCA
H573D-F	CCCGCGCATTCCACCGACCGTGCGACGAGCCA
H573S-F	CCCGCGCATTCCACCTCCCGTGCGACGAGCCA
H573Y-F	CCCGCGCATTCCACCTACCGTGCGACGAGCCA
H573W-F	CCCGCGCATTCCACCTGGCGTGCGACGAGCCA
H573V-F	CCCGCGCATTCCACCGTACGTGCGACGAGCCA
H573M-F	CCCGCGCATTCCACCATGCGTGCGACGAGCCA
H573-R <sup>a</sup>	GGTGGAATGCGCGGGGCTCGCGCCC

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H729R-F	CGCCCGCCGTGCATCCGCCCGGCGAACGCCGT
H729K-F	CGCCCGCCGTGCATCAAGCCGGCGAACGCCGT
H729N-F	CGCCCGCCGTGCATCAACCCGGCGAACGCCGT
H729D-F	CGCCCGCCGTGCATCGACCCGGCGAACGCCGT
H729S-F	CGCCCGCCGTGCATCTCCCGGCGAACGCCGT
H729Y-F	CGCCCGCCGTGCATCTACCCGGCGAACGCCGT
H729W-F	CGCCCGCCGTGCATCTGGCCGGCGAACGCCGT
H729I-F	CGCCCGCCGTGCATCATCCGGCGAACGCCGT
H729M-F	CGCCCGCCGTGCATCATGCCGGCGAACGCCGT
H729-R <sup>a</sup>	GATGCACGGCGGGCGCAAGCCGAAC

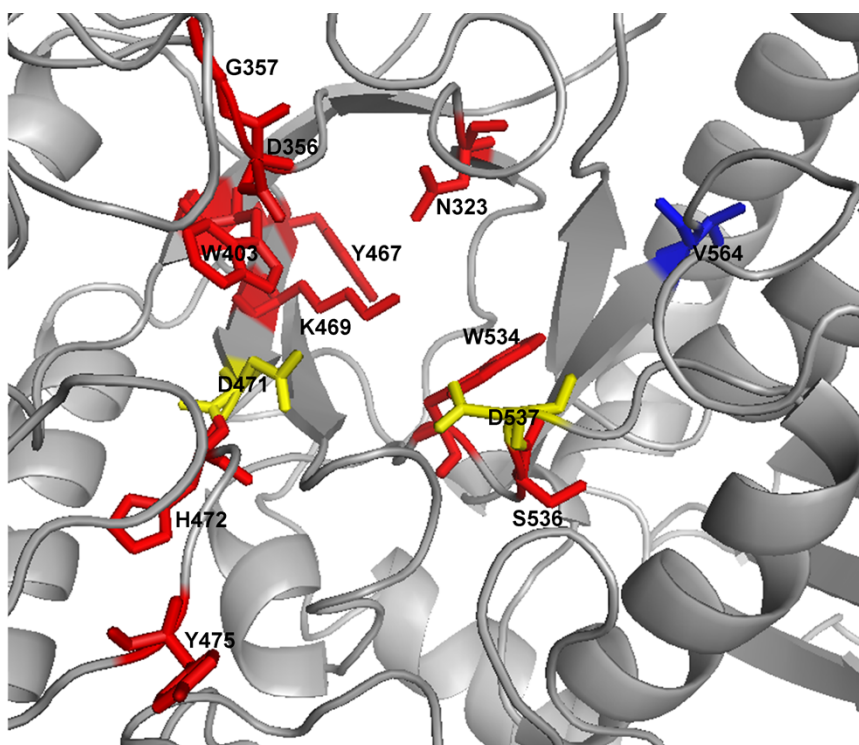
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<sup>a</sup> Mutations occurred at the same site shared one downstream primer.

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## 5. Structures modeling of WT Aga2 and V564N

The model structures of WT Aga2 and V564N were constructed via the Phyre2 server (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>), using  $\alpha$ -galactosidase (PDB ID 3MI6) from *Lactobacillus brevis* as template, with which WT Aga2 shared 35% amino acid sequence identity. The model catalytic center of WT Aga2 was shown in Figure S2, and different kinds of amino acids were labeled with different color.



**Figure S2.** The model catalytic center of WT Aga2. Catalytic residues are yellow; residue V564 is blue; the residues considered to play positive impact on the transglycosylation efficiency are red.

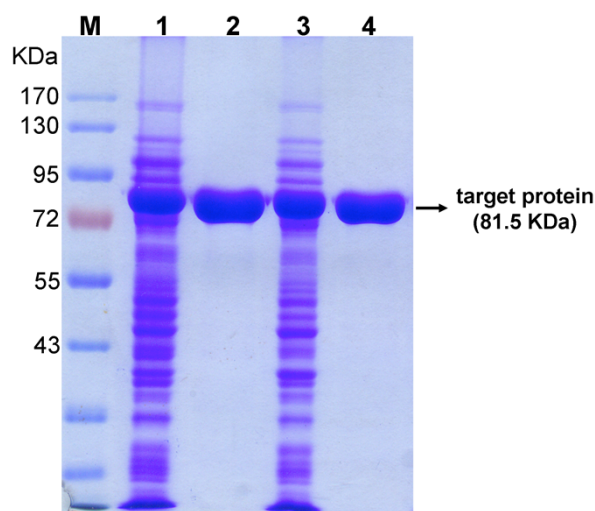
## 6. Protein expression and crude enzyme preparation

The *E. coli* BL21 (DE3) harboring pET-22b-*aga2* or its mutated derivatives was cultured at 37 °C in LB medium containing 50 µg/mL of ampicillin. The protein was induced by the addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) until the culture reached an optical density of 0.6-0.8 at 600 nm, and the culture was incubated overnight at 22 °C. Then cells were harvested by centrifugation (12000 rpm at 4 °C for 5 min), suspended in 50 mM sodium acetate buffer (pH 5.5), and disrupted by sonication (model VCX500; Sonics & Materials, Inc., Newtown, CT) in an ice bath. After centrifugation at 12000 rpm at 4 °C for 25 min, the supernatant was used as crude enzyme.

## 7. Purification of WT Aga2 and V564N

His<sub>6</sub>-tagged proteins of WT Aga2 and V564N were purified from their crude enzymes by nickel affinity chromatography with the ÄKTA purifier 10 system (GE Healthcare). All the

procedures described below were performed in 50 mM phosphate buffer at pH 7.0. The crude enzyme was firstly loaded onto the Ni-Agarose (Ni Sepharose 6 Fast Flow, GE Healthcare) column preequilibrated with binding buffer (20 mM imidazole, 0.5 M NaCl, phosphate buffer). Then, the column was washed with binding buffer and washing buffer (50 mM imidazole, 0.5 M NaCl, phosphate buffer) until the absorbance at 280 nm reached the baseline. The proteins of interest were eluted with eluting buffer (200 mM imidazole, 0.5 M NaCl, phosphate buffer), and the fractions containing the desired protein were pooled. The purified enzymes were concentrated using an ultra centrifugal filter (30 KDa molecular weight cut-off; Millipore; USA). The result of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S3) proved successful purification of WT Aga2 and V564N.



**Figure S3.** SDS-PAGE analysis of WT Aga2 and V564N. Lanes: M, protein standards; 1 and 2, cell lysate and purified protein of WT Aga2; 3 and 4, cell lysate and purified protein of V564N.

## 8. Transglycosylation reaction conditions

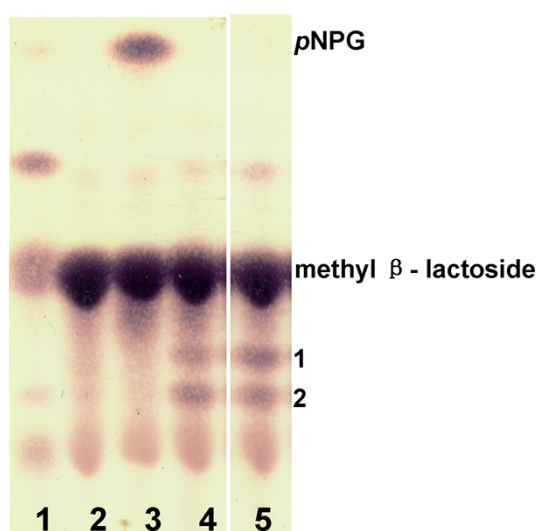
### Melibiose as the sole substrate

The self-transfer reaction using melibiose as the sole substrate for the determination of transglycosylation efficiency was performed at 37 °C with 0.2 M melibiose and 2 U/mL crude enzymes in 50 mM sodium acetate buffer (pH 5.5) for 0.5 h.

### Methyl $\beta$ -lactoside as acceptor

The transglycosylation reactions were performed at 37 °C using 40 mM *p*NPGal as donor

and 0.4 M methyl  $\beta$ -lactoside (Carbosynth, UK) as acceptor with 1 U purified WT Aga2 (12  $\mu\text{g}/\text{mL}$ ) and mutant V564N (37  $\mu\text{g}/\text{mL}$ ) in 200  $\mu\text{L}$  citrate-phosphate buffer (0.1 M citric acid and 0.2 M disodium phosphate, pH 6.5), respectively. TLC results of the reaction mixtures are shown in Figure S4. For the analysis of time course of products, aliquots were withdrawn at proper time intervals during the testing time of 6 h, and the reaction aliquots were boiled for 10 min to inactivate the enzyme. The product yield was determined by HPLC according to the added *p*NPGal. The ratio of the two isomers yields were estimated on the basis of TLC results using the software IMAGEJ 1.40 (<http://rsb.info.nih.gov/ij/>).



**Figure S4.** TLC analysis of the transglycosylation reaction mixtures by WT Aga2 and V564N with methyl  $\beta$ -lactoside as acceptor, respectively. Lanes: 1, reaction including enzyme and donor; 2, reaction including enzyme and acceptor; 3, reaction including inactivated enzyme, donor and acceptor; 4 and 5, reactions catalyzed by Aga2 and V564N respectively.

## 9. Analysis of transglycosylation products by TLC and HPLC

TLC was performed with Silica gel 60 F254 plates (Merck, Germany). The developing solvent was a mixture of butanol-1, ethanol and water (5:3:2, v/v/v). Sugars on the TLC plate were detected by spraying with a solution of 0.5% (w/v) 3, 5-dihydroxytoluene dissolved in 20% (v/v) sulfuric acid and subsequently heating at 120  $^{\circ}\text{C}$  for 5 min.

HPLC was performed on an Agilent 1200 series instrument equipped with an Aminex HPX-42C column (7.8  $\times$  300 mm) using Agilent G1362A refractive index detector. Samples



were eluted with distilled water at a flow rate of 0.4 mL/min, with column oven temperature maintained at 70 °C.

## 10. Isolation of the transglycosylation products

Transglycosylation products were generated by mutant V564N in a 10 mL reaction solution. Following heat inactivation (100 °C, 10 min) and centrifugation (12000 rpm, 20 min), the supernatant was extracted twice with the same volume of diethyl ether to remove by-product *p*-nitrophenol. The aqueous layer was subjected to Bio-gel-P2 (Bio-Rad) column (1.5 × 100 cm) chromatography to isolate the transglycosylation products. The isolated products were further separated by HPLC using a Kromasil Silica column (4.5 × 250 mm) eluted with a mixture of 1-butanol, ethanol and water (5:3:2, v/v/v) at a flow rate of 0.2 mL/min at 20 °C. All of the eluted fractions were analysed by TLC. The fractions with identical compositions were pooled and lyophilized to get a dry powder.

## 11. Identification of transglycosylation products by MS and NMR

Mass spectra (MS) were measured with an API4000 TQ Mass Spectrometer (Applied Biosystems, American) with electronic spray ionization (ESI) of samples. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded at 25 °C on a Bruker DRX Arance 600 MHz spectrometer (Switzerland) at 600 MHz for <sup>1</sup>H and 150 MHz for <sup>13</sup>C. Chemical shifts in parts per million (ppm) were reported relative to the internal standard 2, 2-dimethyl-2-silapentane -5-sulfonate. Chemical shifts were obtained from the analysis of 1D and 2D NMR spectra. Standard homo- and hetero-nuclear correlated 2D NMR techniques, including correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple band correlation (HMBC) experiments, were used to substantiate the assignments.

### **Methyl $\alpha$ -D-Galactopyranosyl-(1→3)- $\beta$ -D-galactopyranosyl-(1→4)- $\beta$ -D-glucopyranoside (*Gal* $\alpha$ 1-3*Gal* $\beta$ 1-4*Glc* $\beta$ OMe, 1)**

ESI-MS: *m/z* [M+Na<sup>+</sup>] 541.2. <sup>1</sup>H NMR (600MHz, D<sub>2</sub>O):  $\delta$  4.99 (d, *J* = 3.6 Hz, 1H), 4.36 (d, *J* = 7.8 Hz, 1H), 4.26 (d, *J* = 8.0 Hz, 1H), 4.05-4.03 (m, 2H), 3.88-3.84 (m, 2H), 3.81 (dd, *J* =

10.8, 2.4 Hz, 1H)), 3.71 (dd,  $J = 10.2, 3.0$  Hz, 1H)), 3.69-3.55 (m, 7H), 3.54-3.44 (m, 4H), 3.43 (s, 3H, OMe), 3.16 (t,  $J = 7.8$  Hz, 1H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  103.00, 102.70, 95.30, 78.40, 77.00, 74.80, 74.50, 74.30, 72.50, 70.60, 69.50, 69.10 (2C), 67.90, 64.60, 60.80, 59.80, 57.20.

**Methyl  $\alpha$ -D-Galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranoside  
(Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc $\beta$ OMe, 2)**

ESI-MS:  $m/z$  [ $\text{M}+\text{Na}^+$ ] 541.2.  $^1\text{H}$  NMR (600MHz,  $\text{D}_2\text{O}$ ):  $\delta$  4.84 (d,  $J = 4.2$  Hz, 1H), 4.32 (d,  $J = 7.8$  Hz, 1H), 4.27 (d,  $J = 7.8$  Hz, 1H), 3.87-3.77 (m, 6H), 3.69-3.64 (m, 2H), 3.62-3.58 (m, 2H), 3.57-3.46 (m, 5H), 3.43 (s, 3H, OMe), 3.44-3.39 (m, 2H), 3.15 (t,  $J = 8.5$  Hz, 1H);  $^{13}\text{C}$  NMR (150 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  103.10, 102.80, 98.20, 79.10, 74.40 (2C), 74.20, 72.60, 72.30, 71.00, 70.60, 68.80, 68.00, 66.20 (2C), 61.00, 59.90, 57.10.

## 12. NMR spectra of compounds 1 and 2

Compound 1, *Gal $\alpha$ 1-3Gal $\beta$ 1-4Glc $\beta$ OMe*

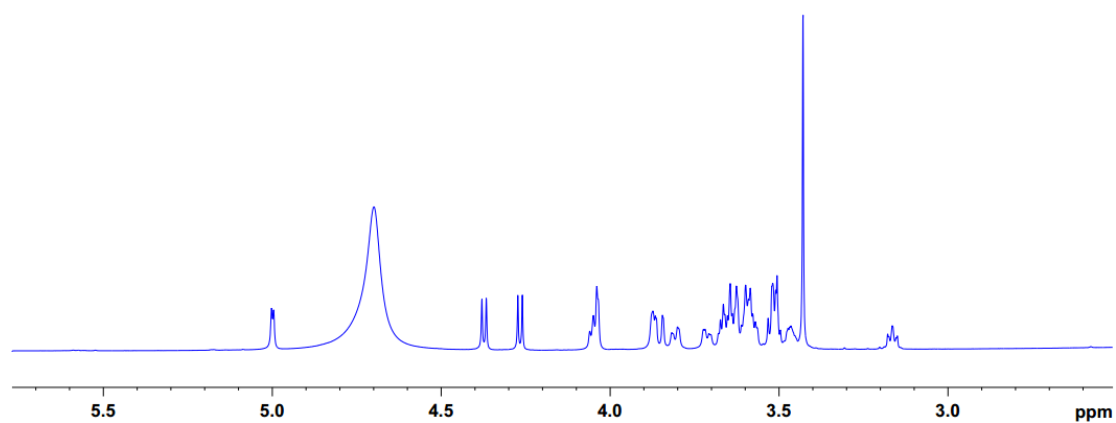


Figure S5. <sup>1</sup>H NMR spectrum of compound 3

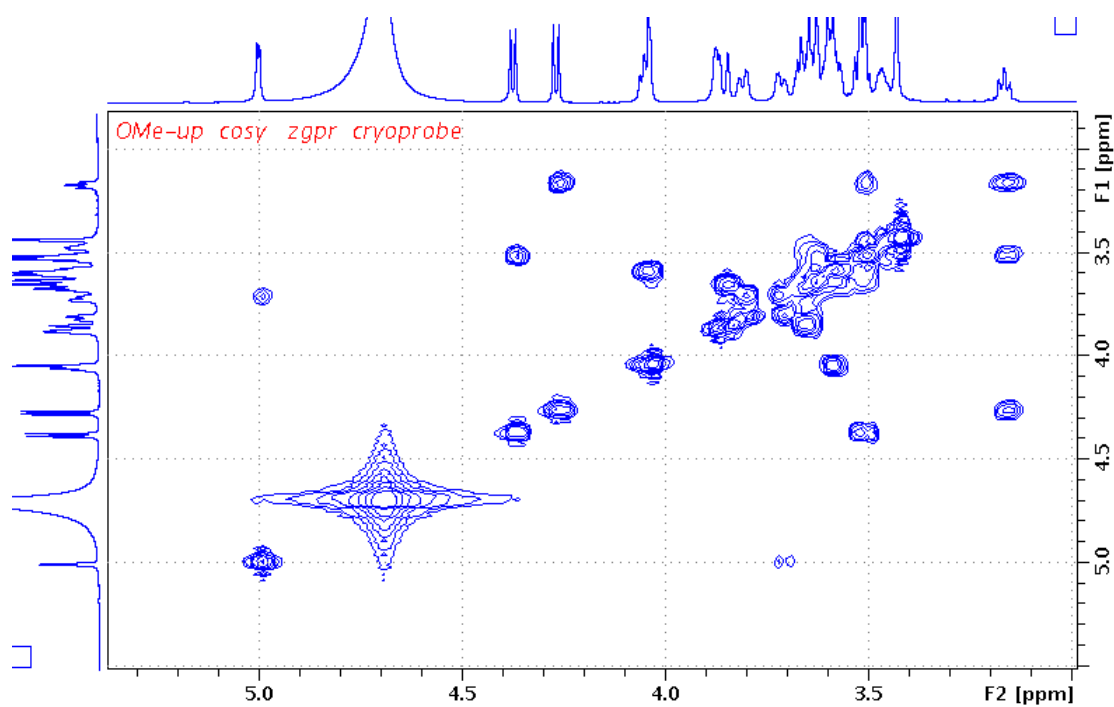


Figure S6. COSY spectrum of compound 3

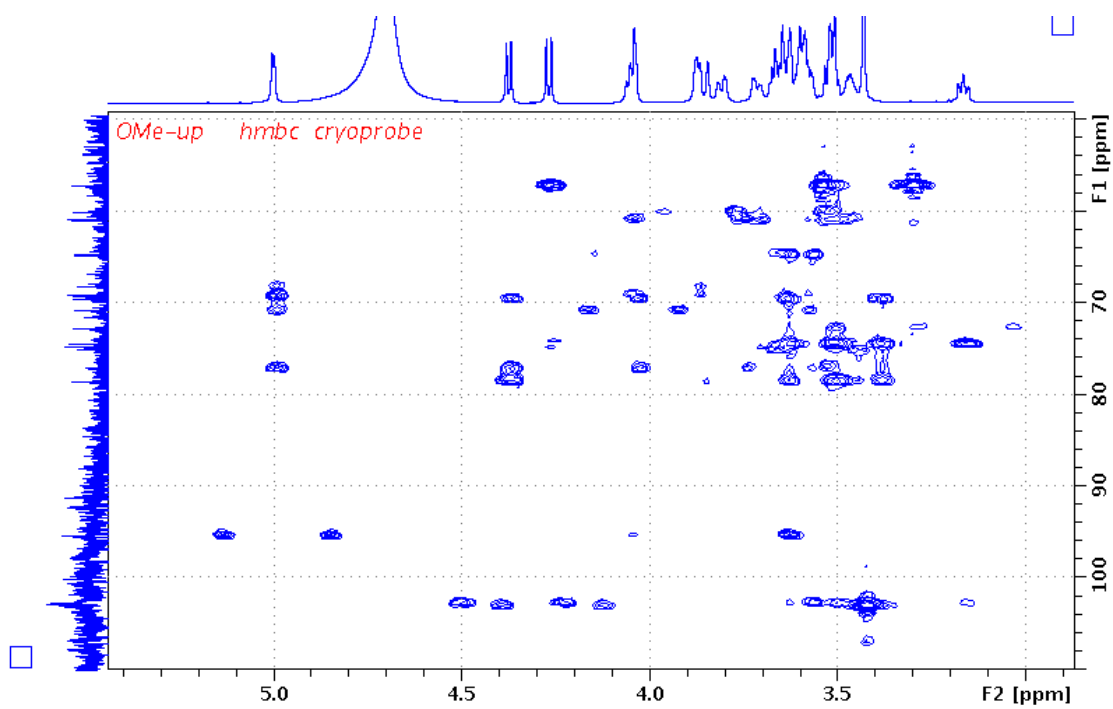
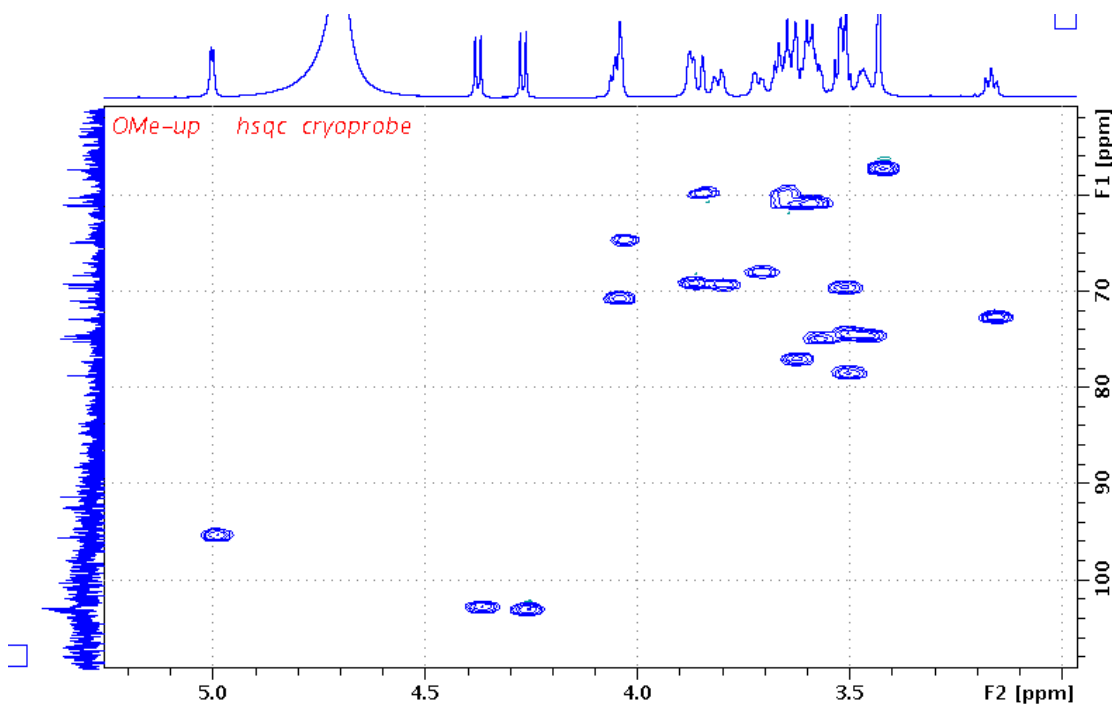
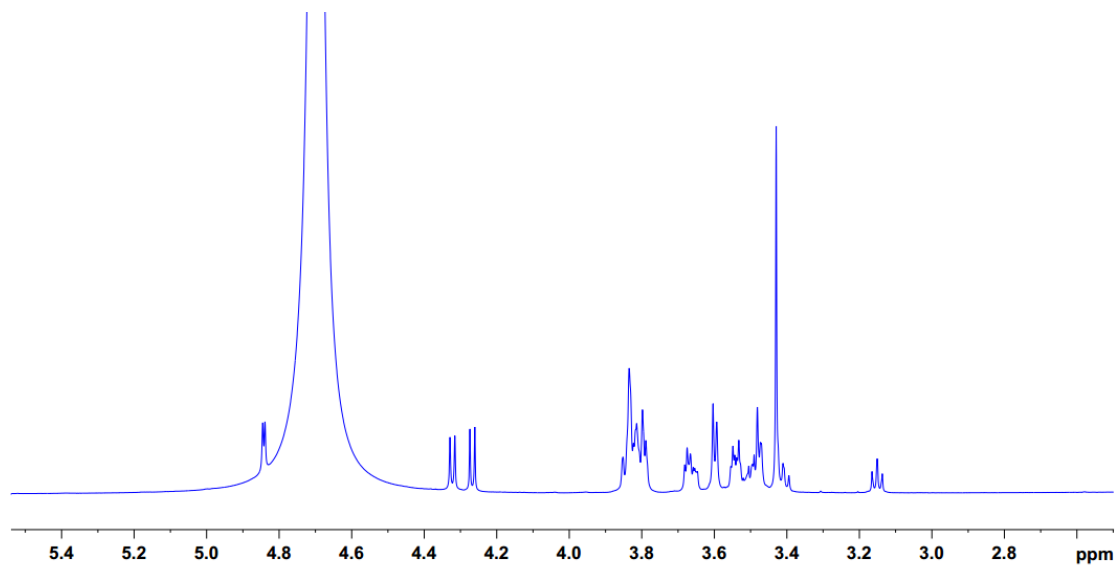


Figure S7. HMBC spectrum of compound 3

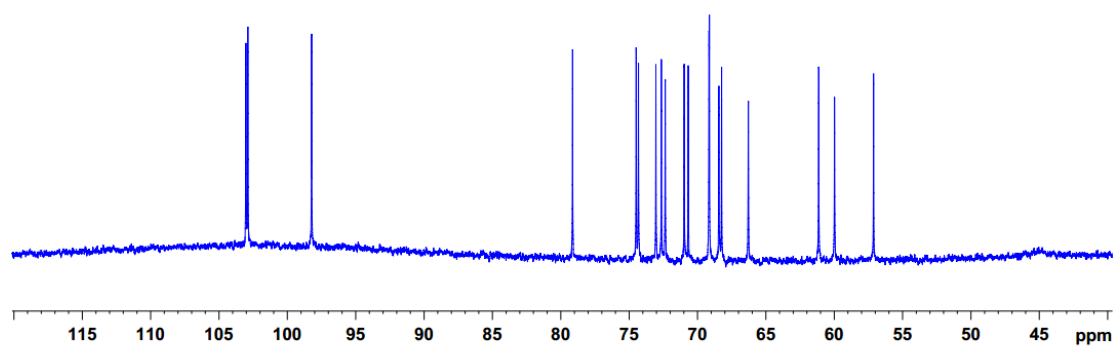


**Figure S8.** HSQC spectrum of compound **3**

Compound **2**, *Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc $\beta$ OMe*



**Figure S9.** <sup>1</sup>H NMR spectrum of compound **4**



**Figure S10.** <sup>13</sup>C NMR spectrum of compound **4**

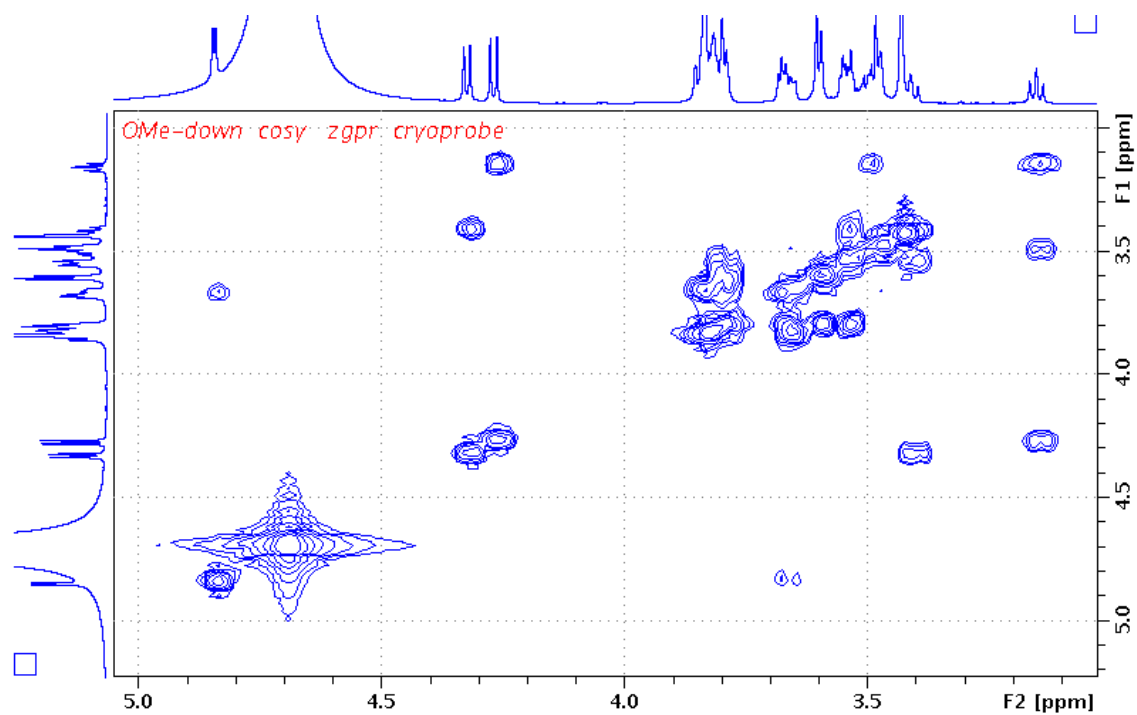


Figure S11. COSY spectrum of compound 4

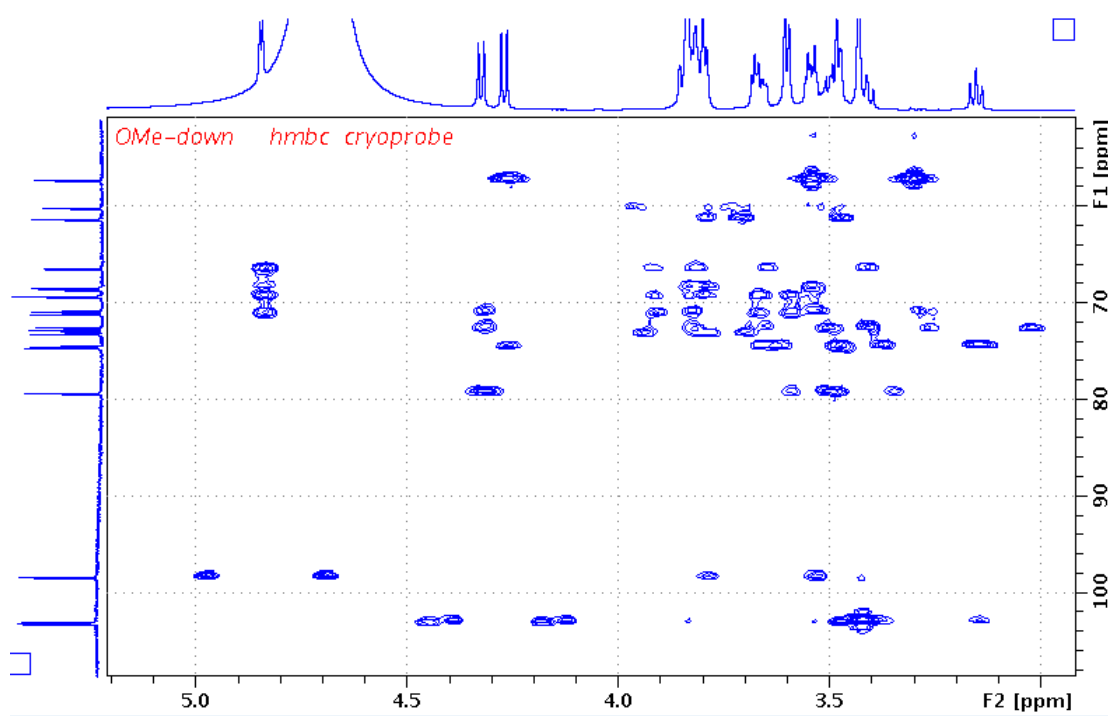


Figure S12. HMBC spectrum of compound 4

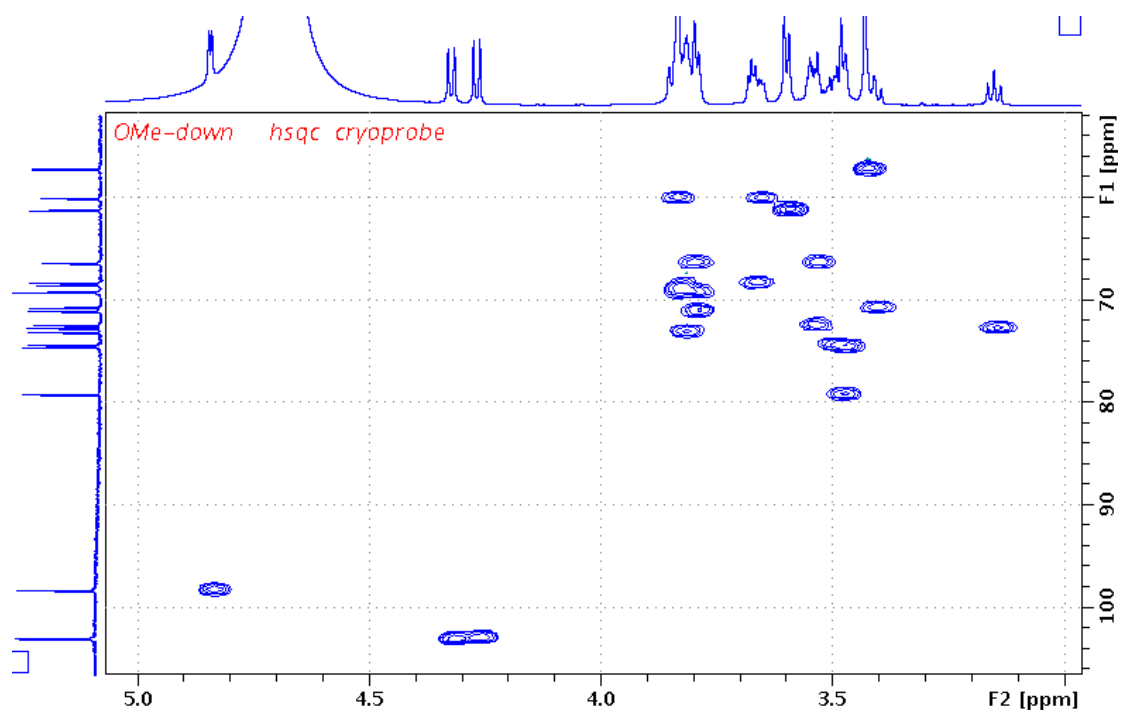


Figure S13. HSQC spectrum of compound 4