



Title	Efficient one-pot enzymatic synthesis of trehalose 6-phosphate using GH65 $\alpha$ -glucoside phosphorylases
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1 **Efficient one-pot enzymatic synthesis of trehalose 6-phosphate using GH65  $\alpha$ -glucoside**  
2 **phosphorylases**

3  
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15  
16 **Abstract**

17 **Trehalose 6-phosphate (Tre6P) is an important intermediate for trehalose**

18 **biosynthesis. Recent researches have revealed that Tre6P is an endogenous signaling molecule**

19 **that regulates plant development and stress responses. The necessity of Tre6P in physiological**

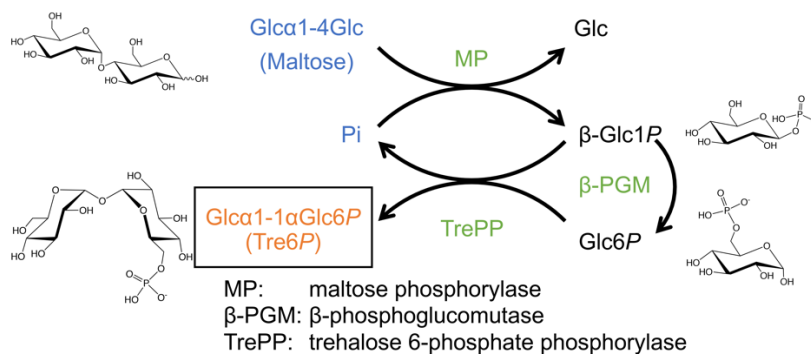
20 **studies is expected to be increasing. To achieve the cost-effective production of Tre6P, a novel**

21 **approach is required. In this study, we utilized trehalose 6-phosphate phosphorylase (TrePP)**

1 from *Lactococcus lactis* to produce Tre6P. In the reverse phosphorolysis by the TrePP, 91.9 mM  
 2 Tre6P was produced from 100 mM  $\beta$ -glucose 1-phosphate ( $\beta$ -Glc1P) and 100 mM glucose 6-  
 3 phosphate (Glc6P). The one-pot reaction of TrePP and maltose phosphorylase (MP) enabled  
 4 production of 65 mM Tre6P from 100 mM maltose, 100 mM Glc6P, and 20 mM inorganic  
 5 phosphate. Addition of  $\beta$ -phosphoglucomutase to this reaction produced Glc6P from  $\beta$ -Glc1P  
 6 and thus reduced requirement of Glc6P as a starting material. Within the range of 20–469 mM  
 7 inorganic phosphate tested, the 54 mM concentration yielded the highest amount of Tre6P (33  
 8 mM). Addition of yeast increased the yield because of its glucose consumption. Finally, from  
 9 100 mmol maltose and 60 mmol inorganic phosphate, we successfully achieved production of  
 10 37.5 mmol Tre6P in a one-pot reaction (100 mL), and 9.4 g Tre6P dipotassium salt was  
 11 obtained.

12

13 Graphical abstract



14

15

1    Highlights

2    Tre6P was formed from  $\beta$ -Glc1P and Glc6P by TrePP.

3    With two other enzymes, Tre6P was produced from only maltose in one-pot reactions.

4    Gram-scale synthesis of Tre6P from maltose and inorganic phosphate was established.

5

6    Keywords

7    Trehalose 6-phosphate; Trehalose 6-phosphate phosphorylase; Glycoside hydrolase family 65;

8    Maltose phosphorylase; Phosphoglucomutase

9

10    *Abbreviations:* COSY, correlated spectroscopy; ESI, electrospray ionization; GH 65, glycoside

11    hydrolase family 65;  $\beta$ -Glc1P,  $\beta$ -D-glucose 1-phosphate; Glc6P, D-glucose 6-phosphate; GP,

12    glycoside phosphorylase; HMBC, heteronuclear multiple bond correlation; HPAEC-PAD, high-

13    performance anion-exchange chromatography equipped with a pulsed amperometric detector;

14    HSQC, heteronuclear single quantum coherence; HSQC-TOCSY, HSQC–total correlation

15    spectroscopy; LITrePP, trehalose 6-phosphate phosphorylase from *Lactococcus lactis* subsp. *lactis*;

16    MS, mass spectrometry; MP, maltose phosphorylase; NMR, nuclear magnetic resonance;  $\beta$ -PGM,  $\beta$ -

17    phosphoglucomutase; TPS,  $\alpha,\alpha$ -trehalose-phosphate synthase; TrePP, trehalose 6-phosphate

18    phosphorylase; Tre6P, trehalose 6-phosphate.

19

20    **1. Introduction**

21    Trehalose ( $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Glcp) is a non-reducing disaccharide, which is widely distributed

22    in diverse organisms, including bacteria, fungi, insects, and plants. In these organisms, trehalose

23    serves as an important compatible solute and stress protectant. Trehalose is synthesized through the

24    Trehalose phosphate synthase–Trehalose phosphatase (TPS–TPP) pathway in various organisms.

1 The first enzyme in the pathway,  $\alpha,\alpha$ -trehalose phosphate synthase (EC 2.4.1.15; TPS), catalyzes the  
2 formation of trehalose 6-phosphate ( $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Glcp6P; Tre6P) from UDP-glucose and  
3 D-glucose 6-phosphate (Glc6P), and the second enzyme, trehalose phosphatase (EC 3.1.3.12; TPP),  
4 dephosphorylates Tre6P [1]. Besides the occurrence of Tre6P as the intermediate in the TPS–TPP  
5 pathway, Tre6P is also produced as a bacterial trehalose metabolite. The bacterial  
6 phosphotransferase system transports extracellular trehalose into cells concomitant with its  
7 phosphorylation [2]. The Tre6P produced is then hydrolyzed by Tre6P hydrolase [3] or  
8 phosphorolyzed into  $\beta$ -D-glucose 1-phosphate ( $\beta$ -Glc1P) and Glc6P by trehalose 6-phosphate  
9 phosphorylase (EC 2.4.1.216; TrePP) [4]. The physiological importance of Tre6P as a signaling  
10 molecule in the regulation of development, stress tolerance, and carbohydrate metabolism in plants  
11 has been recognized in the last decade. The null mutant of *Arabidopsis thaliana tps1*, which is  
12 thought to cause Tre6P deficiency, is unable to complete embryogenesis, resulting in embryonic  
13 lethality [5,6]. The *AtTPS1* overexpressor in *A. thaliana* displays a dehydration tolerance phenotype  
14 [7]. Chemically modified Tre6P, which is permeable in plants, changes sucrose allocation and  
15 increases yield in wheat [8]. These findings indicate that Tre6P is important for plant development  
16 and shows potential for agricultural applications. Not only for the plant, but Tre6P is also good to  
17 target for antibiotic development. In *Mycobacterium tuberculosis*, the gene of TPP which degrade  
18 Tre6P is necessary for growth because of the Tre6P-associated toxicity [9]. Improved access to  
19 Tre6P expected to the development of new antibiotics.

20 To better understand the physiological functions of Tre6P, especially in plants, efficient  
21 synthetic methods are required to produce a sufficient amount of Tre6P for analyses. To date, Tre6P  
22 has been synthesized by yeast fermentation [10] and organic synthesis [11,12]. In the former system,  
23 D-glucose provided as the carbon source is converted into Tre6P with 11% yield. The latter system  
24 produces yields higher than 50% but requires several reaction steps, including protection and

1 deprotection of hydroxy groups, and uses an organic solvent. In contrast, enzymatic synthesis is  
2 simpler and environmentally friendly because it is carried out in aqueous solution under mild  
3 conditions.

4 Glycoside phosphorylases (GPs) catalyze the reversible phosphorolysis of glycosides with  
5 strict substrate specificity. Thus, they are useful catalysts for the specific and efficient synthesis of  
6 oligosaccharides by reverse phosphorolysis. A variety of oligosaccharides have been produced by  
7 glycosyl transfer from glycosyl phosphate in the reverse phosphorolysis of GPs [13,14]. Because the  
8 sugar phosphate can be supplied through the phosphorolysis of abundant oligosaccharides by a  
9 specific GP, a sugar phosphate is not always necessary as a starting material to produce target  
10 oligosaccharides. For example,  $\alpha,\alpha$ -trehalose was produced with a high yield of 60% from the  
11 abundant oligosaccharide maltose and a catalytic amount of inorganic phosphate through  
12 phosphorolysis by maltose phosphorylase (EC 2.4.1.8; MP) and reverse phosphorolysis by trehalose  
13 phosphorylase (EC 2.4.1.64) in a one-pot reaction [15]. Some  $\alpha$ -(1 $\rightarrow$ 4)-glucosyl disaccharides were  
14 also produced with very high yields (84%–91%) from maltose and a number of other sugars by MP  
15 alone [16].

16 The TrePP of *Lactococcus lactis* subsp. *lactis* is thought to be responsible for the  
17 degradation of Tre6P because this bacterium possesses the phosphotransferase system incorporating  
18 trehalose but lacks genes encoding Tre6P-metabolizing enzymes, namely TPP,  $\alpha,\alpha$ -trehalase (EC  
19 3.2.1.28), or  $\alpha,\alpha$ -phosphotrehalase (EC 3.2.1.93) [4]. Its amino acid sequence indicates that TrePP is  
20 a member of the glycoside hydrolase family 65 (GH65), which comprises inverting  $\alpha$ -glucoside  
21 phosphorylases, such as MP,  $\alpha,\alpha$ -trehalose phosphorylase, and kojibiose phosphorylase (EC  
22 2.4.1.230) [17]. TrePP catalyzes the phosphorolysis and synthesis of Tre6P through a sequential bi-bi  
23 mechanism involving the formation of a ternary complex of the enzyme and two substrates [4,18].  
24 The reverse phosphorolysis of TrePP was used to synthesize Tre6P. However, a large amount of

1 starting materials is difficult to use. In this study, we describe a simple and efficient enzymatic  
2 method for Tre6P synthesis from simple abundant carbohydrate material using TrePP in combination  
3 with some other enzymes.

4

## 5 **2. Materials and methods**

### 6 2.1. Chemicals and enzymes

7 As described previously [18],  $\beta$ -Glc1P was prepared from maltose using MP. Recombinant TrePP  
8 from *L. lactis* subsp. *lactis* (LTrePP) was heterologously transformed into BL21 (DE3) competent  
9 *Escherichia coli* cells and purified by Ni-affinity column chromatography as described previously  
10 [18]. One unit of TrePP activity was defined as the amount of enzyme that produces 1  $\mu$ mol  
11 inorganic phosphate from 10 mM  $\beta$ -Glc1P and 10 mM Glc6P per minute under the condition  
12 previously described [18]. MP was purchased from Oriental Yeast (Tokyo, Japan). As stated in the  
13 manufacturer's instructions, 1 unit of MP was defined as the amount of enzyme that produces 1  
14  $\mu$ mol D-glucose per minute from 100 mM maltose and 100 mM phosphate buffer (pH 5.6) at 30°C.  
15  $\beta$ -Phosphoglucomutase (EC 5.4.2.6;  $\beta$ -PGM) was purchased from Sigma-Aldrich (St Louis, MO,  
16 USA) and used to obtain the results in section 3.3. For the other reactions, recombinant  $\beta$ -PGM from  
17 *L. lactis* subsp. *lactis* was used, which was heterologously transformed into BL21 (DE3) competent  
18 *E. coli* cells and purified by Ni-affinity column chromatography. The *pgmB* gene encoding a  $\beta$ -PGM  
19 (GenBank accession no. AE005176.1) was amplified by PCR using genomic DNA of *L. lactis* subsp.  
20 *lactis* JCM 5805 as the template, primers (sense, 5'-  
21 AAAGAAGGTAAAAAGATGTTTAAAGCAGT -3'; antisense, 5'-  
22 TTATTAATTACGAATTATTTTGCTTTTG -3'), and PrimeSTAR<sup>®</sup> HS DNA polymerase (Takara  
23 Bio, Otsu, Japan). Amplified DNA was ligated into the pET23a expression vector (Novagen,  
24 Darmstadt, Germany) at the sites between *NdeI* and the His-tag coding sequence

1 (CACCACCACCACCACCACTGA) using the In-Fusion<sup>®</sup> HD Cloning Kit (Takara Bio). One unit  
2 of  $\beta$ -PGM was defined as the amount of enzyme that converts 1  $\mu$ mol  $\beta$ -Glc1P to Glc6P per minute  
3 at 37°C and pH 7.0 [19]. A hexose phosphatase, YidA, from *E. coli* (EC3.1.3.23; accession no.  
4 NP\_418152.1 [20,21]) was prepared as described previously [20]. One unit of YidA is defined as the  
5 amount of enzyme that releases 1  $\mu$ mol inorganic phosphate from 20 mM Glc6P per minute at 37°C  
6 and pH 7.0.

7

## 8 2.2. Quantification of carbohydrates

9 Carbohydrates were quantified using high-performance anion-exchange chromatography equipped  
10 with a pulsed amperometric detector (HPAEC-PAD ICS 5000<sup>+</sup>; Thermo Fisher Scientific, Waltham,  
11 MA, USA) on a CarboPac PA1 column (4 mm i.d.  $\times$  250 mm; Thermo Fisher Scientific). The mobile  
12 phase was 200 mM NaOH with a linear gradient of sodium acetate (0–250 mM) in 40 min (flow  
13 rate, 0.8 mL/min). The sample injection volume was 10  $\mu$ L. The standards were 25–200  $\mu$ M Tre6P  
14 (Sigma Aldrich), 25–200  $\mu$ M Glc6P (Oriental Yeast), 25–200  $\mu$ M  $\beta$ -Glc1P, 50–400  $\mu$ M maltose  
15 (Nacalai Tesque, Kyoto, Japan), and 50–400  $\mu$ M glucose (Nacalai Tesque). The retention times of  
16 glucose, maltose, Tre6P,  $\beta$ -Glc1P, and Glc6P were 4, 9, 19, 20, and 26 min, respectively.

17

## 18 2.3. Tre6P synthesis from $\beta$ -Glc1P and Glc6P

19 The reaction mixture (10 mL) containing 0.2 U/mL LITrePP, 100 mM  $\beta$ -Glc1P, 100 mM Glc6P, and  
20 250 mM sodium acetate buffer (pH 5.0) was incubated at 30°C for 36 h. The reaction was stopped  
21 by heating the sample at 80°C for 5 min. Inorganic phosphate was measured following the method of  
22 Lowry and Lopez [22]. The concentration of Tre6P after the reaction was determined by HPAEC-  
23 PAD. Remaining  $\beta$ -Glc1P, Glc6P, inorganic phosphate, and sodium acetate were removed from the  
24 sample by electro dialysis with a Microacylizer G1 instrument (Asahi Kasei, Tokyo, Japan) as



1 described previously [18]. Electrospray ionization (ESI)–mass spectrometry (MS) of the  
2 oligosaccharide produced was carried out using an Executive mass spectrometer (Thermo Scientific,  
3 San Jose, CA, USA). The sample was introduced by flow injection. Methanol was used as the  
4 mobile phase solvent. The negative ion was detected under the following conditions: tube lens  
5 voltage, 80 V; skimmer voltage, 30 V. Nuclear magnetic resonance (NMR) spectra were recorded in  
6 D<sub>2</sub>O (99.9%; Sigma) using a Bruker AMX500 instrument (500 MHz, Bruker, Billerica, MA, USA).  
7 A series of two-dimensional homo- and heteronuclear correlated spectroscopy (COSY),  
8 heteronuclear single quantum coherence (HSQC), HSQC–total correlation spectroscopy (HSQC-  
9 TOCSY), and heteronuclear multiple bond correlation (HMBC) spectra were obtained.

10

#### 11 2.4. Tre6P synthesis from maltose and Glc6P using TrePP and MP

12 The reaction mixture (1 mL) containing 1.2 U/mL LITrePP, 3.0 U/mL MP, 100 mM maltose, 100  
13 mM Glc6P, and 20 mM sodium phosphate buffer (pH 6.8) was incubated at 30°C. Aliquots (20 µL)  
14 were taken at indicated times and heated at 80°C for 5 min to stop the reaction. The concentration of  
15 Tre6P after the reaction was determined by HPAEC-PAD.

16

#### 17 2.5. Tre6P synthesis from maltose and inorganic phosphate using TrePP, MP, and β-PGM

18 The reaction mixture (1 mL) containing 1.2 U/mL LITrePP, 3.0 U/mL MP, 1.0 U/mL β-PGM, 100–  
19 120 mM maltose, 20, 54, 110, 224, or 469 mM sodium phosphate, 1 mM magnesium acetate, and 0.1  
20 µM α-D-glucose 1,6-bisphosphate (Sigma) was incubated at 30°C for 120 h. Aliquots (100 µL) were  
21 taken and heated at 80°C for 5 min. β-Glc1P, Glc6P, Tre6P, glucose, and maltose were quantified by  
22 HPAEC-PAD, and inorganic phosphate was measured following the method of Lowry and Lopez  
23 [22].

24

Synthesis of Tre6P from high concentrations of the substrates (1 M maltose and 0.6 M

1 sodium phosphate buffer, pH 7.0) was performed in 1 mL reaction mixture. This reaction mixture  
2 contained the same concentrations of enzyme and magnesium acetate but did not contain  $\alpha$ -D-  
3 glucose 1,6-bisphosphate. Efficiency improvement was achieved by addition of 1% (w/v) bakery  
4 yeast [23,24]. The reaction proceeded under the same condition. Compounds were quantified as  
5 described above. On day 4 of the reaction, 20.6  $\mu$ L of the enzyme mixture containing 1.2 U LITrePP,  
6 3.0 U MP, and 3.0 U  $\beta$ -PGM was added, and the incubation was continued.

7 For Tre6P preparation, 100 mL of the reaction mixture containing 1 M maltose, 0.6 M  
8 sodium phosphate buffer (pH 7.0), 1 mM magnesium acetate, and 3 U/mL MP was incubated at  
9 30°C for 5 days. At 48 h, 2 mL of the enzyme mixture containing 320 U LITrePP, 500 U MP, and  
10 590 U  $\beta$ -PGM was added. The reaction was terminated on day 5 by heating at 80°C for 5 min. To  
11 remove Glc6P and  $\beta$ -Glc1P, 2.0 U/mL YidA was added and incubated at 30°C for 8 h followed by  
12 heating at 80°C for 5 min. The reaction mixture was filtered and glycine (Wako Pure Chemical  
13 Industries, Osaka, Japan) was added to a final concentration of 1 M. This mixture was concentrated  
14 at 60°C in a rotary evaporator for the Maillard reaction. The sample was loaded onto an anion  
15 exchange column of Amberjet® 4400 resin (4.8 cm i.d.  $\times$  30 cm, 300 mL, hydroxy form, Organo,  
16 Tokyo, Japan) in accordance with a previously described method [25]. Fractions containing Tre6P  
17 were collected, and magnesium acetate and ammonia were added (12 mmol, three times the  
18 inorganic phosphate content) for struvite precipitation [26,27]. The pH was adjusted to 10 with 6 M  
19 KOH, and the mixture was concentrated in a rotary evaporator. The mixture was subjected to gel  
20 filtration chromatography on a TOYOPEARL® HW-40S column (5.0 cm i.d.  $\times$  100 cm, 1770 mL;  
21 Tosoh, Tokyo, Japan) using water as the eluent (flow rate: 0.9 mL/min). Fractions containing Tre6P  
22 were collected, and the pooled sample was passed through a column of cation exchange resin  
23 (Dowex 50, H<sup>+</sup> type; Wako Pure Chemical Industries) to remove the counterions of Tre6P. The pH  
24 was adjusted to 8.5 with 6 M KOH and concentrated in a rotary evaporator. Then Tre6P was

1 precipitated in 96% ethanol. The purity was confirmed by HPAEC-PAD and NMR.

### 3. Results and discussion

#### 3.1. Tre6P synthesis from $\beta$ -Glc1P and Glc6P by reverse phosphorolysis of LITrePP

5 LITrePP was incubated with 100 mM  $\beta$ -Glc1P and 100 mM Glc6P at 30°C, and released inorganic  
6 phosphate was monitored (Fig. 1). The concentration of inorganic phosphate was identical to that of  
7 the produced Tre6P. Tre6P did not significantly change after 24 h. Tre6P attained 91.9 mM in 36 h.  
8 From this result, the equilibrium constant of the phosphorolysis of Tre6P — $K_{eq} = [\beta\text{-Glc1P}][\text{Glc6P}]/$   
9  $[\text{Tre6P}][\text{inorganic phosphate}]$ — was determined to be 0.0078. The reaction product was purified by  
10 electro dialysis and analyzed by ESI-MS and NMR. The product gave a signal at 421.07  $m/z$  [M]<sup>-</sup>.

11 The chemical shifts detected in the <sup>1</sup>H- and <sup>13</sup>C-NMR analyses are summarized in Table 1.

12 Correlation peaks between C1 of Glc and H1 of Glc6P, and between H1 of Glc and C1 of Glc6P,  
13 were observed in the HMBC spectrum, which indicated that double glucosidic linkages had formed  
14 between Glc and Glc6P. The  $J_{H1, H2}$  values of Glc and Glc6P were 3.75 Hz and 3.85 Hz,  
15 respectively, which indicated that both pyranoses were in an  $\alpha$ -anomeric configuration. The chemical  
16 shifts obtained from the <sup>1</sup>H- and <sup>13</sup>C-NMR matched those previously reported for Tre6P [11,12].

17 Consequently, the reaction product of LITrePP from  $\beta$ -Glc1P and Glc6P was confirmed to be Tre6P.

#### 3.2. One-pot synthesis of Tre6P from maltose and Glc6P by LITrePP and MP

20 Instead of using  $\beta$ -Glc1P as a starting material,  $\beta$ -Glc1P synthesis from maltose, which is a readily  
21 available substrate, was investigated. Maltose and Glc6P were used as starting materials for Tre6P  
22 synthesis. It was expected that  $\beta$ -Glc1P was supplied by phosphorolysis of maltose by MP and used  
23 for Tre6P production by LITrePP (Fig. 2A). The inorganic phosphate used as a substrate in the first  
24 MP reaction is released in the second reverse phosphorolysis. Using 100 mM maltose and 100 mM

1 Glc6P in the presence of 20 mM sodium phosphate buffer (pH 6.8) as initial concentrations, Tre6P  
2 was produced and accumulated to 65 mM in 28 h (Fig. 2B). Extending the reaction time after 8 h did  
3 not significantly change the Tre6P concentration. The yield was 65% based on the maltose  
4 concentration (100 mM) and comparable to those of trehalose production from maltose (60%) by the  
5 action of two  $\alpha$ -glucoside phosphorylases, trehalose phosphorylase and MP [15].

6

7 3.3. One-pot synthesis of Tre6P from only maltose and its dependence on inorganic phosphate  
8 concentration

9 Tre6P was produced using maltose as a sole carbohydrate source. The expected reaction scheme is  
10 shown in Fig. 3A. Compared with the reaction shown in Fig. 2A, Glc6P was not added, but the  
11 addition of  $\beta$ -PGM was expected to compensate by isomerization of  $\beta$ -Glc1P. This enzyme has strict  
12 anomeric selectivity and it did not produce  $\alpha$ -glucose 1-phosphate. Tre6P was produced from 100–  
13 120 mM maltose and 20–469 mM inorganic phosphate. The progress of other reaction products was  
14 monitored (Fig. 3B–F). During all the reactions, the change in the amounts of total glucosyl groups  
15 and total phosphate in the reaction mixtures was within 12%. In the reaction with the initial  
16 inorganic phosphate concentration of 20 mM (Fig. 3B), the products attached a plateau rapidly (in 6  
17 h). The concentration of Tre6P was 20 mM, which was comparable to the initial concentration of  
18 inorganic phosphate, and those of  $\beta$ -Glc1P and Glc6P were low, which indicated that almost all the  
19 inorganic phosphate was incorporated into Tre6P. The Tre6P yield based on the initial concentration  
20 of maltose (100 mM) was 20%; that is, 40% of maltose molecules were used for Tre6P synthesis.

21 Hereinafter, the percentage of maltose molecules that was used for its synthesis was used as the  
22 yield. The  $K_{eq}$  ( $K_{eq} = K_{TrePP} \times K_{MP}^2 \times K_{\beta-PGM} = [Tre6P][glucose]^2 / ([maltose]^2[inorganic phosphate])$ )  
23 calculated at 12 h was  $3.87 \pm 0.18$ , which is close to the theoretical  $K_{eq}$  (4.73), calculated from  $K_{TrePP}$   
24 (31.3 at 35°C in pH 7.0 [4]),  $K_{MP}$  (0.071 at 30°C in pH 7.0 [28]),  $K_{\beta-PGM}$  (30 at 30°C in pH 7.0; data

1 not shown). Higher initial inorganic phosphate concentrations up to 224 mM resulted in higher  
2 consumption of maltose up to 93% consumption (Fig. 3E). The maximum Tre6P concentration (33  
3 mM) was attained with 48 h reaction with an initial phosphate concentration of 54 mM (Fig. 3C).  
4 The yield was 55%. Initial phosphate concentrations higher than 54 mM resulted in lower Tre6P  
5 production but significant accumulation of Glc6P. The ratio of produced Tre6P and Glc6P was not  
6 changed by decreasing  $\beta$ -PGM concentration, which only delayed the reaction attaining the plateau  
7 (data not shown). It is preferable for efficient Tre6P production to set the concentration of inorganic  
8 phosphate lower than that of maltose. In the 120 h reaction with 469 mM inorganic phosphate, lower  
9 consumption of maltose (59% consumption), a higher concentration of  $\beta$ -Glc1P than Glc6P, and  
10 lower production of Tre6P (15 mM) were observed (Fig. 3F). In the early stage of all reactions,  
11 maltose decreased quickly. Concomitantly,  $\beta$ -Glc1P was accumulated first and replaced by Tre6P  
12 and Glc6P. The reduction of  $\beta$ -Glc1P was delayed depending on inorganic phosphate concentration.  
13 Unlike other reactions,  $\beta$ -Glc1P was accumulated more than Glc6P under 469 mM inorganic  
14 phosphate. The concentrations continued to change after 80 h and seemed not to attain equilibrium.  
15 We confirmed that under a high concentration of inorganic phosphate, the  $\beta$ -PGM reaction took  
16 longer to attain equilibrium than at a low concentration of inorganic phosphate (data not shown). The  
17  $\beta$ -PGM reaction is considered to be inhibited by inorganic phosphate, which is consistent with the  
18 finding that  $\beta$ -PGM activity of *Euglena gracilis* was inhibited by 20% in the presence of 10 mM  
19 inorganic phosphate [29]. Therefore, the production of Glc6P and Tre6P did not proceed until the  
20 concentration of inorganic phosphate decreased and the  $\beta$ -PGM reaction started. That was the reason  
21 why all reactions took a long time despite the high enzyme concentration.

22

### 23 3.4. Efficiency improvement of Tre6P synthesis using yeast

24 The reaction from a higher concentration of starting materials, 1 M maltose and 0.6 M inorganic

1 phosphate, was examined (Fig. 4A). During the reaction, the change in the amounts of total glucosyl  
2 groups and total phosphate was less than 10%. Maltose concentration decreased up to 800 mM and  
3  $\beta$ -Glc1P was accumulated in the early stage of the reaction in the presence of remaining inorganic  
4 phosphate (Fig. 4A). The maltose and  $\beta$ -Glc1P concentrations did not change after 2 days. That was  
5 because, in the presence of a high concentration of inorganic phosphate, the activity of  $\beta$ -PGM was  
6 inhibited by remained inorganic phosphate, and  $\beta$ -PGM might be deactivated during incubation.  
7 Therefore, we added three enzymes, LITrePP, MP, and  $\beta$ -PGM on day 4. After the supplemental  
8 addition of LITrePP, MP, and  $\beta$ -PGM, maltose and  $\beta$ -Glc1P concentrations decreased, and Tre6P and  
9 Glc6P concentrations increased. At day 9, 0.7 M maltose was consumed, and 0.7 M glucose and 0.3  
10 M Tre6P were produced. The reaction progressed more quickly with addition of yeast in the reaction  
11 mixture for reduction of glucose, which is a product of maltose phosphorolysis (Fig. 4B). The  
12 amounts of total glucosyl groups were decreased to 50% in the 9-days reaction because yeast  
13 consumed liberated glucose. The amount of total phosphate in the reaction mixture was within a  
14 variation of 10%. In this case, maltose and inorganic phosphate concentrations decreased more  
15 quickly and  $\beta$ -Glc1P accumulated to 0.5 M at day 2. After the addition of LITrePP, MP, and  $\beta$ -PGM,  
16 the Tre6P concentration attained 0.4 M in the reaction mixture (the yield was 80%). In either case,  
17 Glc6P and Tre6P were considered not to be produced before the additional supplementation of  
18 enzymes. This is because of the inactivation of  $\beta$ -PGM during the reaction.

19 For preparation of Tre6P, the reaction mixture was up-scaled to 100 mL, which contained  
20 100 mmol maltose, 60 mmol inorganic phosphate, and MP in the presence of yeast (Fig. 5, Table 2).  
21 Because only MP reaction proceeded for 2 days (Fig. 5), the other two enzymes were not added. At  
22 day 2 of incubation, LITrePP, MP, and  $\beta$ -PGM were added. At day 5, Tre6P attained 370 mM (38  
23 mmol). For purification of Tre6P, Glc6P and  $\beta$ -Glc1P were dephosphorylated by a hexose  
24 phosphatase, EcYidA. Remaining glucose, Glc6P were removed by a Maillard reaction followed by

1 anion exchange column chromatography. Glc6P and Tre6P were difficult to separate by anion  
2 exchange column chromatography, but anion exchange column chromatography could remove the  
3 product of Maillard reaction. After this purification procedure,  $\beta$ -Glc1P was not detected. Remaining  
4 inorganic phosphate was removed by struvite precipitation. Then, Tre6P was further purified by gel  
5 filtration and cation exchange column chromatography, and 19 mmol (9.4 g) Tre6P was obtained as  
6 dipotassium salt with a yield of 38%. The purity was higher than 98%. We confirmed the NMR  
7 spectra of purified Tre6P (Fig.6B). These spectra were well-matched with the Tre6P synthesized  
8 from  $\beta$ -Glc1P and Glc6P (Fig.6A).

9           The Tre6P yield of this enzymatic method was higher than that of the yeast fermentation  
10 method (11%) and lower than that of organic synthesis (55%) [10–12]. However, the primary  
11 advantage of the enzymatic synthesis method established here is the simple synthesis of Tre6P at  
12 gram-scale in one-pot reactions from abundantly available starting materials, i.e. maltose and  
13 inorganic phosphate. This synthesis pathway breaks the barrier of Tre6P usage and is expected to  
14 facilitate functional analysis of Tre6P. Tre6P metabolism is a good target for antibiotics for bacteria,  
15 especially *M. tuberculosis* [9].

16           In plants, Tre6P is recognized as an essential signaling molecule in the regulation of  
17 growth and development. Analyses are required for an improved understanding of the functions of  
18 Tre6P. The simple enzymatic synthesis of Tre6P established in this study provides large amounts of  
19 Tre6P for such analyses. Although it may be challenging, isotopically labeled Tre6P, which would  
20 be helpful for tracing metabolites, might be produced by using  $^{13}\text{C}$ -labeled maltose and  $^{32}\text{P}$ -labeled  
21 inorganic phosphate, for instance, as substrates. Tre6P analogs, such as Glc $\alpha$ 1- $\alpha$ 1Man6P [18], also  
22 could be supplied.

23

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3

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10

#### 11 **Declaration of interest**

12 No potential conflict of interest was reported by the authors.

13

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19

## 20 **Figure legends**

21 **Fig. 1. Time course of Tre6P formation from 100 mM  $\beta$ -Glc1P and 100 mM Glc6P by LITrePP.**

22 (A) Expected reaction scheme. LITrePP, Tre6P phosphorylase from *L. lactis* subsp. *lactis*.

23 (B) Time course of accumulation of Tre6P. Starting materials were Glc6P (100 mM),  $\beta$ -Glc1P (100

24 mM). Liberated inorganic phosphate was measured following the method of Lowry and Lopez [22].

1 At 36 h, Tre6P was quantified by HPAEC-PAD. The concentration of inorganic phosphate was  
2 identical to that of the produced Tre6P. Data are means of triplicate experiments with standard  
3 deviations in three independent reactions.

4  
5 **Fig. 2. One-pot synthesis of Tre6P from maltose and Glc6P by LITrePP and MP.**

6 (A) Expected reaction scheme. LITrePP, Tre6P phosphorylase from *L. lactis* subsp. *lactis*;  
7 MP, maltose phosphorylase. (B) Time course of accumulation of Tre6P. Starting materials were  
8 Glc6P (100 mM), maltose (100 mM), and 20 mM inorganic phosphate. Tre6P was quantified by  
9 HPAEC-PAD. Data are means of triplicate experiments with standard deviations in three  
10 independent reactions.

11  
12 **Fig. 3. Production of Tre6P using maltose as a single carbohydrate source.**

13 (A) Expected reaction scheme. An additional enzyme,  $\beta$ -phosphoglucomutase ( $\beta$ -PGM),  
14 was added. (B–F) Time course of accumulation of Tre6P synthesized from 100–120 mM maltose  
15 and inorganic phosphate (B: 20 mM, C: 54 mM, D: 110 mM, E: 224 mM, F: 469 mM). Tre6P and  
16 the other carbohydrates were quantified. Maltose (filled square), inorganic phosphate (open square),  
17 Tre6P (filled circle), glucose (open circle), Glc6P (open triangle), and  $\beta$ -Glc1P (filled triangle). [Pi]:  
18 right side axis in E and F. Data are means of triplicate experiments with standard deviations in three  
19 independent reactions.

20  
21  
22 **Fig. 4. Time course of Tre6P synthesis from 1 M maltose and 0.6 M inorganic phosphate**

1 Time course of Tre6P synthesis reactions without yeast (A) and with yeast (B) are shown.  
2 The enzymes were supplemented at day 4. Symbols are the same as shown in Fig. 3B. Data are  
3 means of triplicate experiments with standard deviations in three independent reactions.

4

5 **Fig. 5. Time course of large-scale (100 mL) synthesis of Tre6P**

6 Tre6P was synthesized in a 100-mL reaction mixture containing 1 M (100 mmol) maltose,  
7 0.6 M (60 mmol) sodium phosphate was monitored. The enzymes were supplemented at day 2.  
8 Symbols are the same as shown in Fig. 3B.

9

10 **Fig.6. <sup>1</sup>H- and <sup>13</sup>C-NMR spectra of synthesized Tre6P**

11 The synthesized Tre6P from (A) β-Glc1P and Glc6P, and (B) maltose and inorganic  
12 phosphate were analyzed by <sup>1</sup>H- and <sup>13</sup>C- NMR.

13

1 **Table 1 Chemical shifts of  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Glcp6P in the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra.**

Residue	Number	$\delta\text{C}$ (ppm)	$\delta\text{H}$ (ppm)	$J$ (Hz)	
$\alpha$ -Glcp	1	94.3	5.27	d	3.75
	2	71.9	3.70	m	
	3	73.4	3.93	m	
	4	70.6	3.51	t	9.43
	5	73.1	3.88	m	
	6	61.5	3.93	m	
			3.83	m	
$\alpha$ -Glcp6P	1	94.4	5.25	d	3.85
	2	72.1	3.76	m	
	3	73.2	3.90	m	
	4	70.1	3.66	t	9.60
	5	72.5	3.97	m	
	6	63.9	4.08	m	

2

3

4

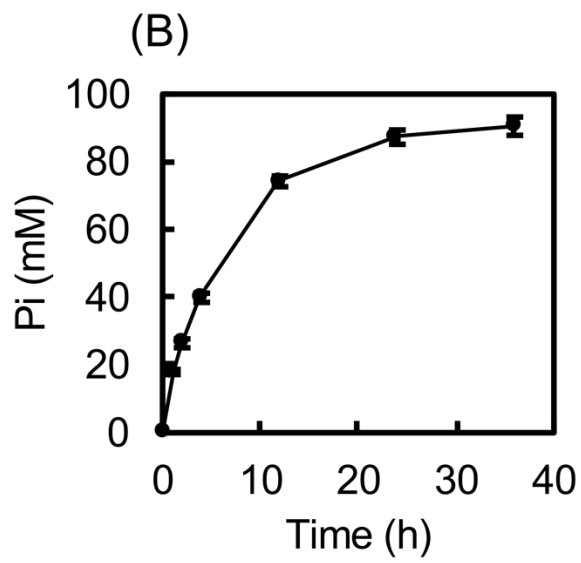
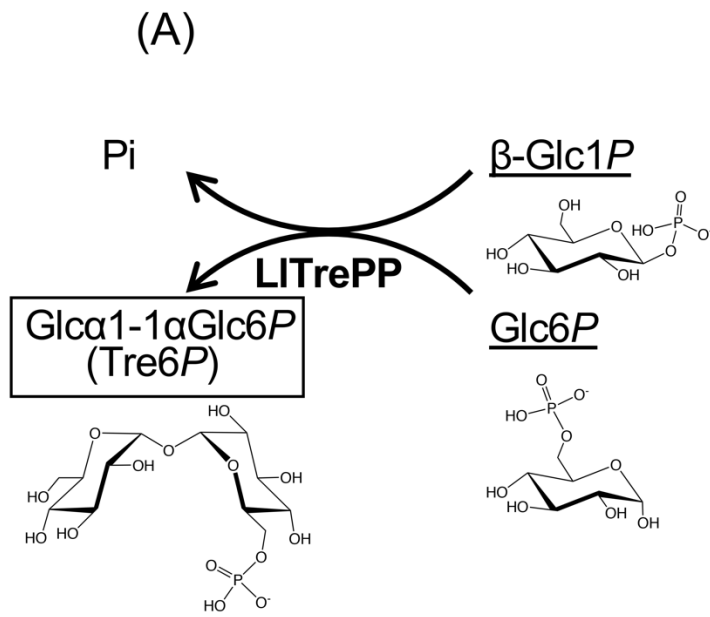
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1 **Table 2 Purification of Tre6P**

Process	Tre6P mmol	Glc6P mmol	$\beta$ -Glc1P mmol	Purity %	Yield %
Reaction	38	12	0.23	64	75
EcYidA	36	8.5	0.19	73	72
Anion-exchange column chromatography	25	-	-	86	50
Gel-filtration column chromatography	19	-	-	98	38

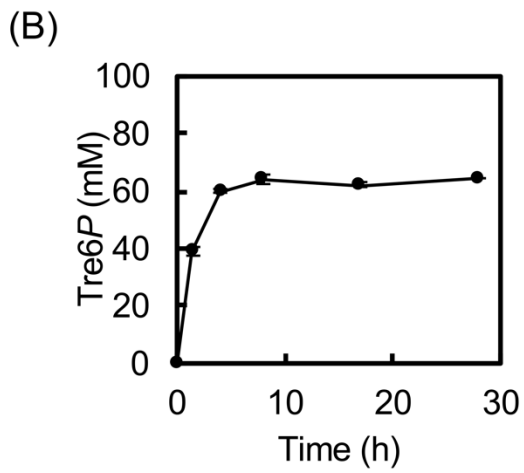
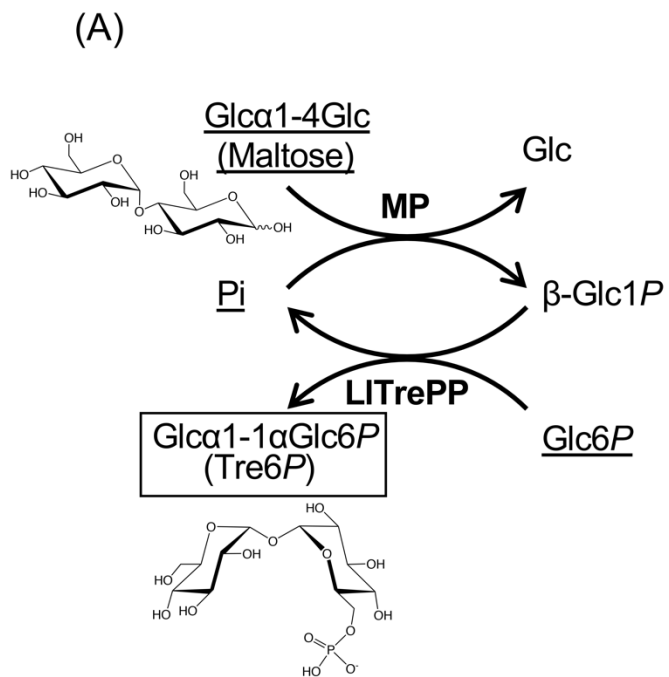
2 \*The yield was calculated based on the maltose molecules used for Tre6P synthesis.

3

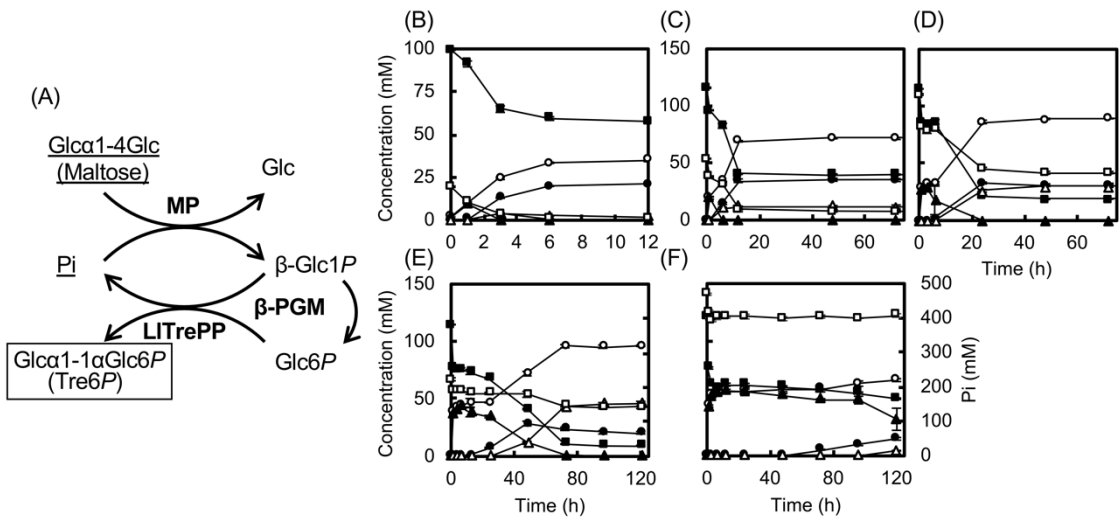


1  
2 Fig. 1





1  
2 **Fig. 2**

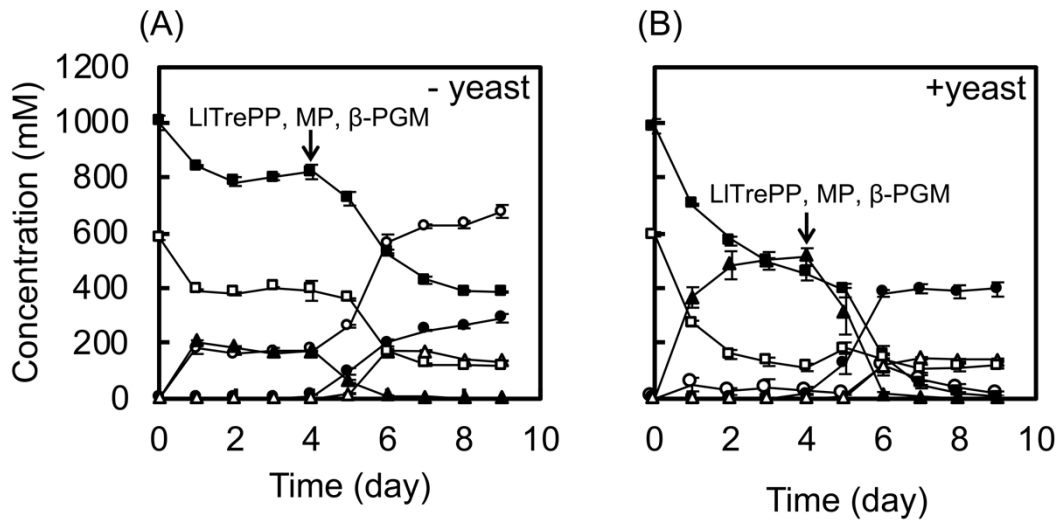


1

2 **Fig. 3**

3

1



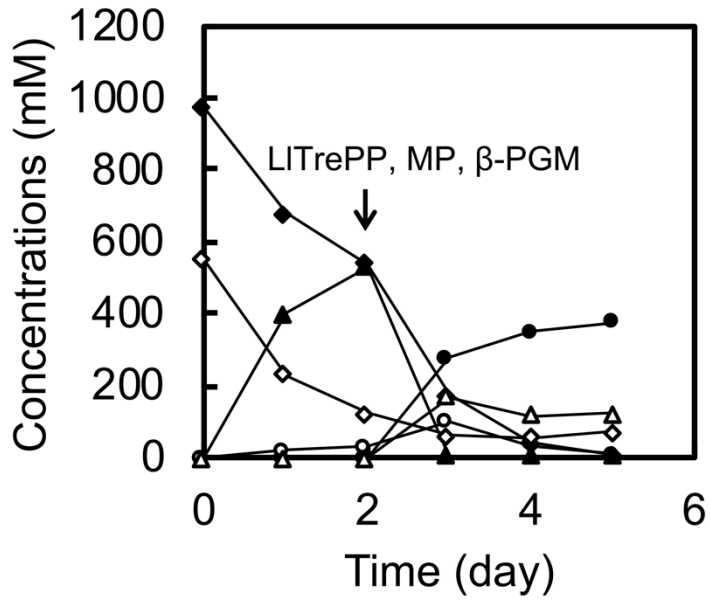
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Fig. 4

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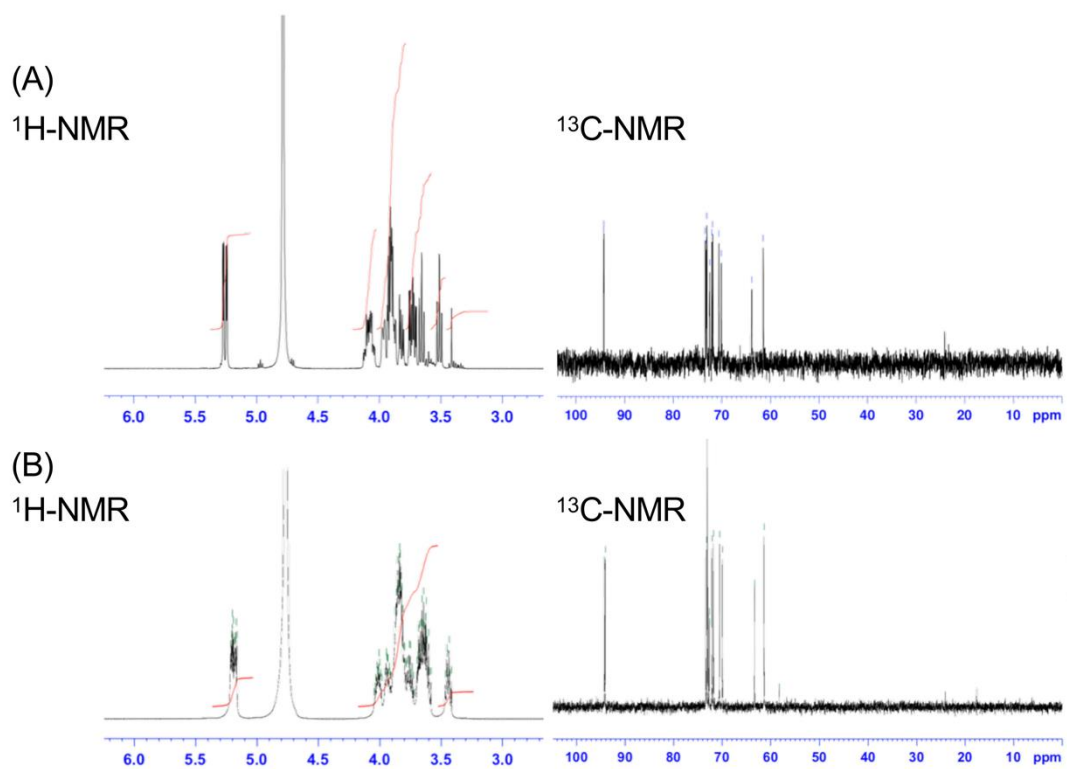
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2

3 **Fig. 5**

4



1

2 **Fig. 6**