Electronic Supplementary Information

Efficient synthesis of ponasterone A by recombinant Escherichia coli harboring the glycosyltransferase GT_{BP1} with in situ product removal

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1 Materials

1.1 Biological and chemical materials

Ponasterone A was purchased from Chemical Solutions Corporation (Fraserton Court, Burnaby, BC, Canada). Ponasteroside A was isolated from rhizomes of *Brainea insignis*, a Chinese traditional medicine. The solvents used in the high-performance liquid chromatography (HPLC) analysis were of HPLC grade from Sigma (USA). Other solvents and reagents were of analytical grade from commercial sources.

1.2 Media

Screening medium consisted of 0.1% ponasteroside A, 0.05% yeast extract, 0.1% NaCl, and 0.05% $MgSO_4$ (pH 7.0). Luria-Bertani (LB) medium consisted of 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, and 1.8% agar powder (pH 7.0). Basic medium consisted of 0.1% rhizomes of *Brainea insignis* extract, 0.8% peptone, 1.5% sucrose, 0.1% KH_2PO_4, and 0.05% $MgSO_4$ (pH 7.0).

2 Experimental

2.1 Isolation of microorganisms with the ability to transform ponasteroside A into ponasterone A

Soil samples were collected from a *Brainea insignis* garden. A small amount of soil was suspended in sterilized distilled water, and 100 μL of the resulting suspension was added to the screening medium. Ponasteroside A was added to the medium as a carbon source. Cultivation was conducted in 250-mL Erlenmeyer flasks containing 50 mL screening medium at 30°C. Incubation was carried out with agitation at 200 rpm for 24 h. Cultures were then acclimated by repeated transfer under the same culture conditions. After three cycles, the cultures were diluted and spread

on plates with Luria-Bertani (LB) medium. The growing colonies were further purified by repeated streaking.

The isolated strains were cultured separately in the basic medium. All cultures were incubated in 50 mL medium (250-mL flasks) at 30°C with shaking at 200 rpm for 12 h. Cells were harvested to catalyze the transformation of ponasteroside A according to the method described in section 2.7. The supernatants of reaction solutions were analyzed by HPLC. A strain with the ability to transform ponasteroside A into ponasterone A was selected and identified based on 16S rDNA sequence analysis in BLAST in the Genebank Data Library.

2.2 Effect of the inhibitors on deglycosylation of ponasteroside A

To investigate the enzyme responsible for the deglycosylation of ponasteroside A, glycosyltransferase inhibitors (hexadecylpyridinium chloride [final concentration, 4.2 mM], hexadecyltrimethyl ammonium bromide [4.1 mM], and sodium dodecyl sulfate [17.3 mM]) and β-glycosidase inhibitors (imidazole [4.6 mM], benzimidazole [6.5 mM], and 2-aminopyridine [16.0 mM]) were used in the biotransformation system containing ponasteroside A and cells of strain BF1. After 24 h, 100 μL transformation liquid was mixed with 900 μL methanol and centrifuged. The supernatant was collected for the assay by HPLC.

2.3 Cloning, expression and purification of the glycosyltransferase GT_{BP1} from *Bacillus pumilus* BF1

Genomic DNA from strain BF1 was obtained using a DNA Purification Wizard Genomic kit (Promega, Madison, WI, USA). Since the strain BF1 shared 99% identity with the typical strain *Bacillus pumilus* MTCC B6033 based on the 16S rDNA sequence alignment, oligonucleotide primers of the glycosyltransferase gene gt_{BP1} were designed based on the gene sequence of the glycosyltransferase in strain *Bacillus pumilus* MTCC B6033 (GenBank accession number: CP007436). The primers (forward: 5′-CGCGAATTCATGGCGAAGGTTTTAATGATTACG-3′, and reverse: 5′-ATACTCGAGTTATGATTGAGTCTGTTTCGCTTGC-3′) were synthesized (restriction enzyme cutting sites are indicated with underlined text). The PCR product was digested and inserted into pET-28a. The gt_{BP1} gene was sequenced and deposited in GenBank with the accession number KX523795.1. The recombinant plasmid pET-28a-gt_{BP1} was transformed into *E. coli* BL21 (DE3).

The recombinant *E. coli* BL21/pET-28a-gt_{BP1} (abbreviated as *E. coli*/gt_{BP1}) was grown in LB

medium with kanamycin (50 μg/mL) at 37°C with shaking. When the OD_{660} of culture reached 0.8, 1 mM isopropyl thio-β-D-galactoside (IPTG) was added, and the cultures were incubated for 6 h at 30°C. The cells were then harvested for sonication. The supernatant was collected by centrifugation at 13,323 g for 30 min at 4°C and analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels.

To conduct the purification, the cells were harvested via centrifugation at 13,323 × g for 30 min, washed two times with buffer (20 mmol/L PBS of pH 7.0) and resuspended in loading buffer (20 mmol/L PBS, 0.1 mol NaCl, 10% glycerol and 50 mmol imidazole of pH 7.0). After the cells were sonicated, the supernatant was isolated by centrifugation at 13,323 × g for 15 min at 4 °C. Subsequently, the glycosyltransferase was purified through His-tag protein purification with standard Ni-NTA resin.

2.4 Quantitative Analysis of substrate and product by HPLC

HPLC (LC-20AT; Shimadzu, Japan) analysis of both substrate and product was performed with an ODS-BP column (4.6 mm × 250 mm, 5 μm; Dalian Elite Analytical Instruments Co., Ltd.). The column temperature was maintained at 25°C. Ultraviolet (UV) detection was set at 230 nm, and the injection volume was 20 μL. The mobile phase was methanol/water (60:40, v/v) at a flow rate of 1.0 mL/min. The linear regression equation for ponasteroside A was Y = 808040x+3219 (R^2 = 0.9995), and Y = 1031768x + 15915 (R^2 = 0.9994) for ponastone A, where Y means peak area and x means the mass (μg) of ponasteroside A and ponastone A. The concentrations of product and substrate were detected in buffer and organic solvent layer respectively, and the results were calculated as total concentrations in buffer, which were calculated by the following formula.

$$C_{Tot}=(C_{Buf}\times V_{Buf}+ C_{Org}\times V_{Org})/V_{Buf} \quad (1)$$

C_{Buf} - the concentration of product or substrate in the buffer phase

V_{Buf} - the volume of the buffer phase

C_{Org}- the concentration of product or substrate in organic solvent phase

V_{Org}- the volume of the organic solvent phase

2.5 NMR analysis

^{1}H NMR spectra were obtained using a Bruker AV-300 spectrometer operating at 300 MHz. Samples were dissolved in DMSO-d_6 at room temperature, and tetramethylsilane (Me_4Si) was used as the chemical shift reference.

2.6 ESI$^+$ TOF MS analysis

The molecular weights of the transfer products were determined using a TOF mass spectrometer (Micromass) equipped with an electrospray ion source. Spectra were obtained in the positive-ion mode.

2.7 The solubility of the substrate and product in the aqueous phase and organic phase

The ponasterone A or ponasteroside A was constantly added to 10 mL of PBS or ethyl acetate until the ponasterone A or ponasteroside A was dissolved. The remained ponasterone A and ponasteroside A were weighted, respectively. The solubilities of the substrate and product in the aqueous phase and organic phase were calculated, respectectively.

2.8 Enzyme assay

The glycosyltransferase $GT_{BP1}$was added to 50 mM Na2HPO4/KH2PO4 buffer (phosphate-buffered saline [PBS]; pH 7.0), containing 0.48 mM ponasteroside A and 0.48mM uridine diphosphate (UDP). Activity of the glycosyltransferase $GT_{BP1}$was based on the increase of ponasterone A. The enzyme reaction was conducted at 30°C, and the reaction solution was evaluated by HPLC. One unit of ponasteroside A glycosyltransferase activity was defined as the amount of enzyme that produced 1 μmol of ponasterone A per minute.

2.9 Bioconversion of ponasteroside A to ponasterone A by *E. coli/gt*$_{BP1}$

E. coli/gt$_{BP1}$ was added to 50 mM PBS, containing 0.48 mM ponasteroside A with a final OD_{660} of about 10 as a reflection of the cell density. The bioconversion was conducted at 30°C in shaking flasks at 200 rpm, and the reaction solution was evaluated by HPLC.

2.10 Effect of organic solvents on the enzyme activity and stability

The effect of solvents with log P values ranging from 0.16 to 2.2 on the enzyme activity and stability were investigated. A double volume of organic solvents was added to 50 mM PBS containing ponasteroside A (0.48 mM), UDP (0.48 mM) and the glycosyltransferase GT_{BP1}. The enzyme activity was measured as described above.

Two milliliters of diluted purified glycosyltransferase were incubated in the absence or presence of 4.0 mL of organic solvent at 30 °C for 1h. The residual activities were determined by the procedure described above.

2.11 Preparation of ponasterone A in a buffer system and aqueous-organic biphasic system

A double volume of organic solvents was added to 100 mL PBS (50 mM) containing ponasteroside A (0.48 mM) and the cells of *E. coli*/gt_{BP1} at final OD_{660} of 10. Bioconversion in the aqueous-organic biphasic system was carried out at 30°C in shaking flasks at 200 rpm.

2.12 Preparation of ponasterone A by fed-batch strategy

The reaction was carried out by keeping the concentration of substrates below 0.5 mM through fed-batch cultivation with the substrate every 20h. A double volume of organic solvents was added to 100 mL PBS (50 mM) containing ponasteroside A (0.48 mM) and the cells of *E. coli*/gt_{BP1} at final OD_{660} of about 10. After 5 batches of reaction, the recombinant *E. coli*/gt_{BP1} was added to the buffer phase with final OD_{660} of 10.

3 Effect of organic solvents on the enzyme activity and stability

The effect of solvents with log P values ranging from 0.16 to 2.2 on the enzyme activity and stability were investigated. The results were listed in Table S1.

Table S1 Effect of organic solvents on the enzyme activity and stability

Organic solvent	Relative activity (%)	Remain activity(%)
Buffer	100	99
Ethyl acetate	377.3	76.4
Menthyl acetate	355.1	54.2
Methyl propionate	363.7	48.7
n-Butyl acetate	376.6	47.7
n-Pentyl acetate	372.1	48.3
Isobutanol	2.2	57.5
Dichloromethane	99.3	65.5

4 The structure of the product

4.1 HPLC Spectra

Fig. S1 represented the sample of different reaction time and the standard of product and substrate.

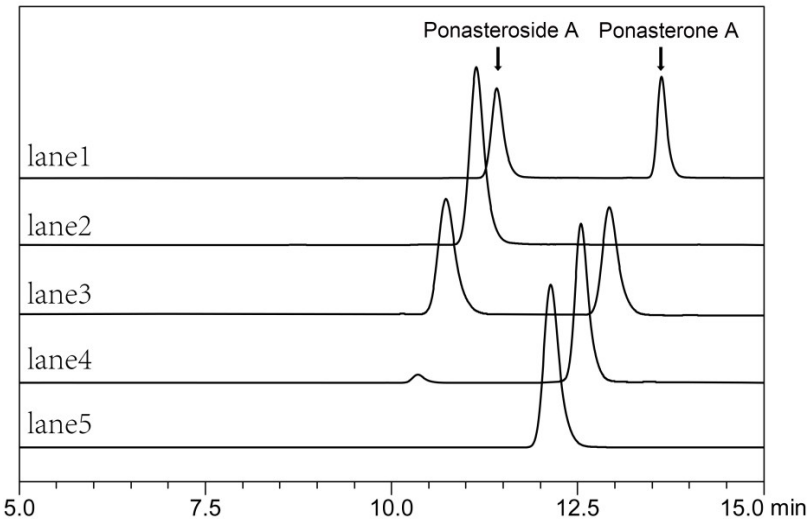
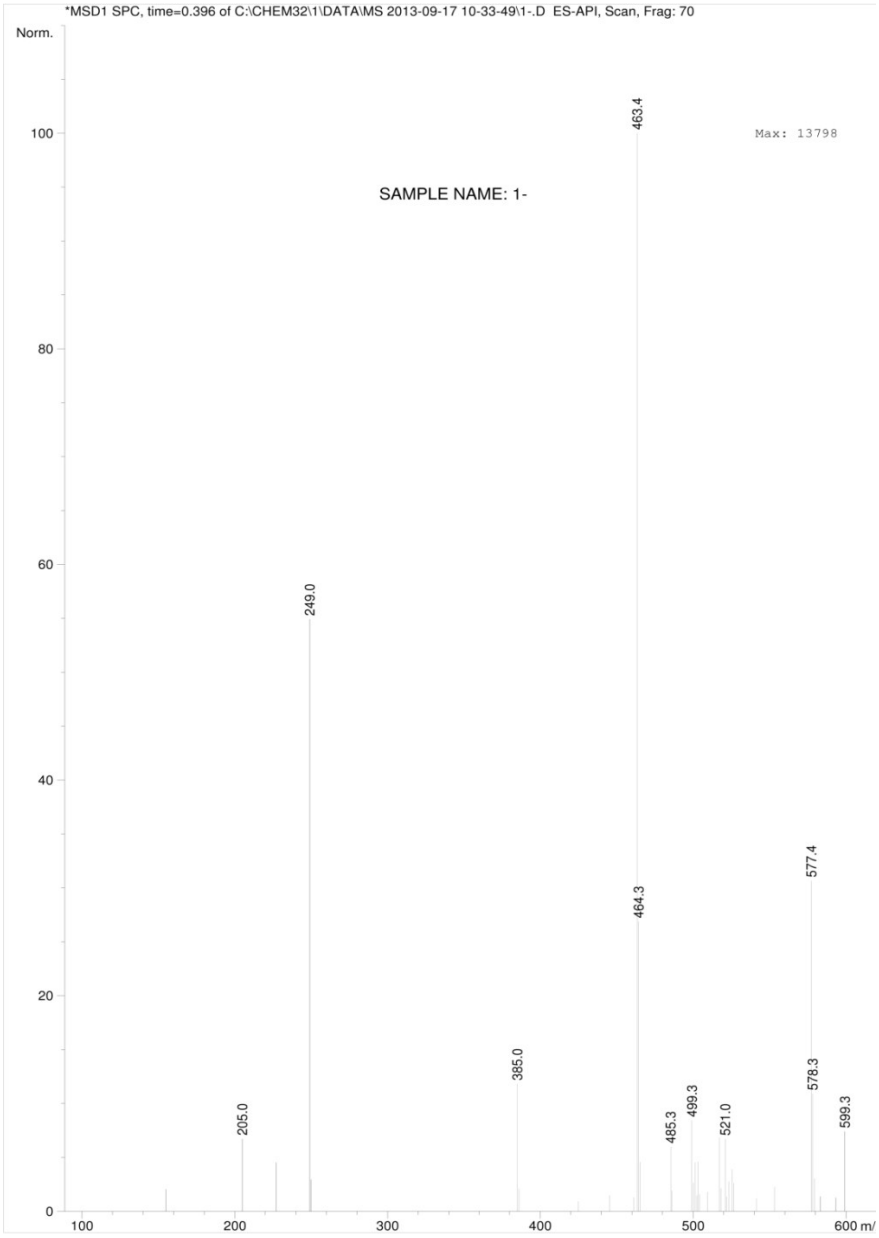


Fig. S1 HPLC analysis of the standard of substrate,product and the reaction process. Lane1: the standard of ponasteroside A and ponasterone A, Lane2: the 0th h sample of the reaction, Lane3: the 8th h sample of the reaction, Lane4: the 20th h sample of the reaction, Lane5: the 28th h sample of the reaction

4.2 NMR and MS

The product was confirmed by mass spectrometry (MS) and (NMR) analysis: MS-ESI (*m/z*) 465.3$[M+H]^+$, 487.2$[M+Na]^+$. ^{1}H NMR (300 MHz, DMSO) δ 5.62 (d, *J* = 1.6 Hz, 1H), 4.65 (s, 1H), 4.43 (d, *J* = 5.9 Hz, 1H), 4.37 (s, 1H), 4.35 (d, *J* = 3.4 Hz, 1H), 3.76 (td, *J* = 6.2, 2.1 Hz, 1H), 3.62 (dd, *J* = 2.5, 1.1 Hz, 1H), 3.59 (s, 1H), 3.13 (dd, *J* = 8.9, 5.3 Hz, 1H), 3.00 (ddd, *J* = 10.7, 7.2, 2.0 Hz, 1H), 2.29 – 2.24 (m, 1H), 2.20 (dd, *J* = 13.4, 4.9 Hz, 1H), 2.12 – 1.92 (m, 2H), 1.92 – 1.76 (m, 2H), 1.76 – 1.66 (m, 2H), 1.66 – 1.59 (m, 2H), 1.59 – 1.52 (m, 2H), 1.53 – 1.48 (m, 2H), 1.48 – 1.43 (m, 1H), 1.43 – 1.34 (m, 2H), 1.34 – 1.19 (m, 2H), 1.19 – 1.07 (m, 2H), 1.05 (s, 3H), 0.88 (s, 3H), 0.86 (d, *J* = 5.6 Hz, 3H), 0.84 (d, *J* = 5.6 Hz, 3H), 0.76 (s, 3H). The NMR spectra of product corresponds with the report of Simon[1].



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152 **Fig. S2. Mass spectrum of ponasterone A.**

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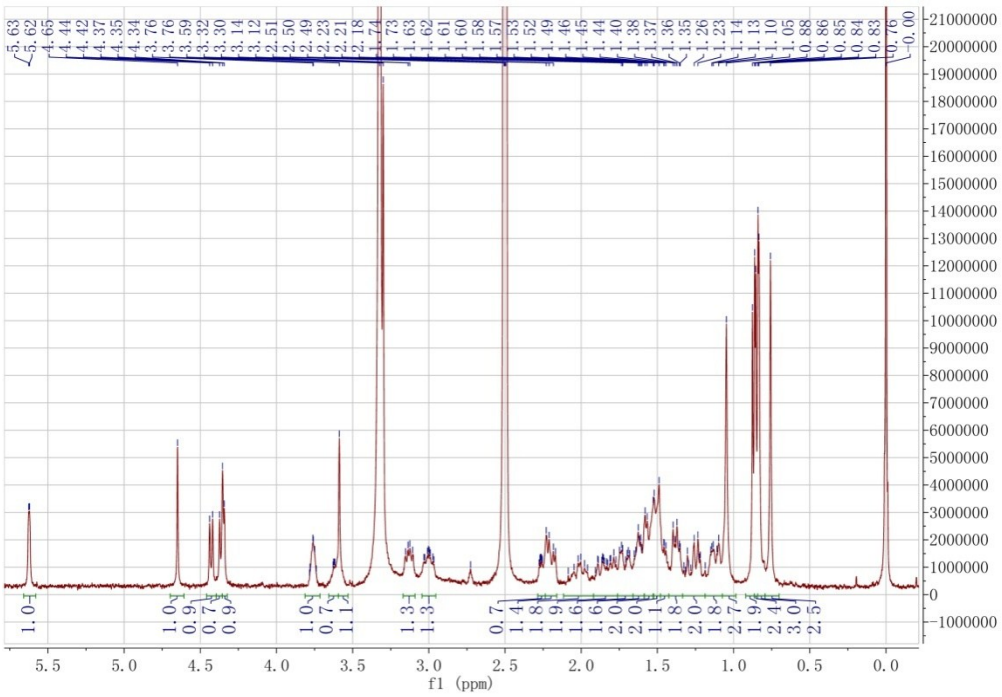

Fig. S3 Nuclear magnetic resonance of ponasterone A.

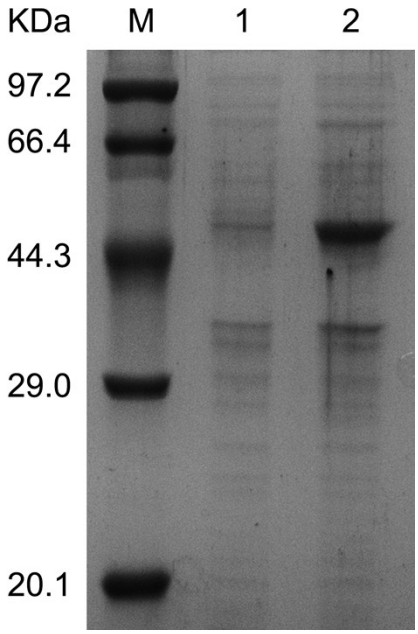

Fig. S4 SDS–PAGE analysis of recombinant glycosyltransferase GT_{BP1}. Lane M, molecular marker; lane 1, lysate of *E. coli*/pET-28a; lane 2, lysate of *E. coli/p*ET-28a-*gt*BP1.

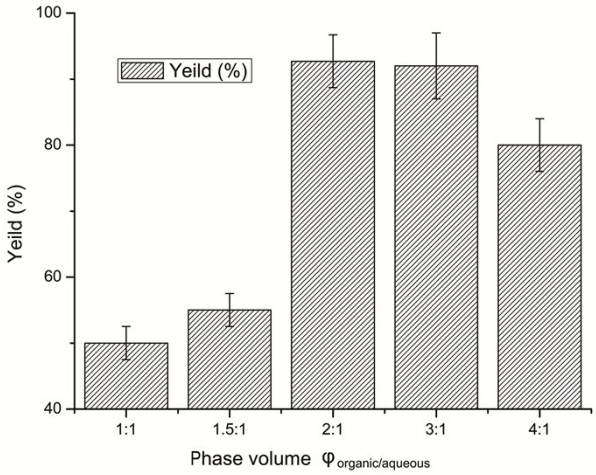

Fig. S5 effect of phase volume ratio on the total yield.

Reference

1. A. Simon, E. Liktor-Busa, G. Tóth, Z. Kele, J. Groska and M. Báthori, *Helvetica Chimica Acta*, 2010, **91**, 1640-1645.