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ORIGINAL RESEARCH PAPER

Heterologous biosynthesis of triterpenoid dammarenediol-II in engineered *Escherichia coli*

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Abstract
Objectives To achieve heterologous biosynthesis of dammarenediol-II, which is the precursor of dammarane-type tetracyclic ginsenosides, by reconstituting the 2,3-oxidosqualene-derived triterpenoid biosynthetic pathway in *Escherichia coli*.
Results By the strategy of synthetic biology, dammarenediol-II biosynthetic pathway was reconstituted in *E. coli* by co-expression of squalene synthase (SS), squalene epoxidase (SE), NADPH-cytochrome P450 reductase (CPR) from *Saccharomyces cerevisiae*, and SE from *Methylococcus capsulatus* (McSE), NADPH-cytochrome P450 reductase (CPR) from *Arabidopsis thaliana*. Sequences of transmembrane domains were truncated if necessary in each of the genes. Different sources of SE/CPR combinations were tested, during which two CPRs were detected to be new reductase partners of McSE. When the gene encoding dammarenediol-II synthase was co-expressed with the 2,3-oxidosqualene expression modules, dammarenediol-II was detected and the production was 8.63 mg l^{-1} in *E. coli* under the shake-flask conditions.
Conclusions Two *E. coli* chassis for production of dammarenediol-II were established which could be potentially applied in other triterpenoid production in *E. coli* when different oxidosqualene cyclases (OSCs) introduced into the system.

Dashuai Li and Qiang Zhang have contributed equally to this work.

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Introduction

Ginsenosides are the major pharmacologically-active constituents of *Panax ginseng*. They are triterpenoids that exhibit potential effects on the central nervous, cardiovascular, endocrine and immune systems (Christensen 2009), in which dammarenediol-II-derived, dammarane-type tetracyclic ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rh2 and Rg3) are the major constituents, while Ro is an oleanane-type pentacyclic ginsenoside in *Panax ginseng* (Han et al. 2011). Currently, ginsenosides are mainly produced by extraction from ginseng roots which is expensive, time-consuming and labour intensive and produce relatively low yields. For these reasons, the engineering of microbial systems to produce ginsenosides or precursors presents an attractive alternative to extractions from environmental sources.

Microbial production of ginsenosides has been realized in *Saccharomyces cerevisiae* (Dai et al. 2013; Yan et al. 2014), attributed to the identification of genes involved in ginsenosides biosynthesis pathway (Tansakul et al. 2006; Han et al. 2011). Also, *Escherichia coli,* as another commonly used recombinant microbial system, has the ability to generate valuable natural products (Jiang et al. 2012; Yin et al. 2015). So heterologous biosynthesis of ginsenosides or precursors in *E. coli* presents an attractive system, because *E. coli* has been genetically and physiologically characterised and multiple genetic engineering tools exist.

The metabolic pathway for terpenoid synthesis consists of an upstream isoprenoid pathway that is native to *E. coli* and a heterologous downstream terpenoid pathway (Ajikumar et al. 2010). Triterpenoids are synthesized from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) via the 30-carbon intermediate squalene and comprise a large group of natural products, then completely identical to each other up to the formation of 2,3-oxidosqualene and branch at its cyclization step (Phillips et al. 2006). The first step in the heterologous terpenoid pathway in *E. coli* is the conversion of farnesyl pyrophosphate (FPP) into squalene by squalene synthase (SS) (Katabami et al. 2015), and the second is the epoxidation of squalene to 2,3-oxidosqualene, which is carried out by the squalene epoxidase (SE), a microsomal protein which requires the participation of the flavoprotein NADPH-cytochrome P450 reductase (CPR) (Sakakibara et al. 1995). The next committed step in triterpenoid biosynthesis is the cyclisation of 2,3-oxidosqualene, this reaction is catalysed by specific oxidosqualene cyclases (OSCs), e.g., dammarenediol-II synthase (DS), β-amyrin synthase (bAS), lupeol synthase (LUS) and lansterol synthase (LAS) (Phillips et al. 2006) as shown in Fig. 1. There are, however, no reports about the formation of 2,3-oxidosqualene or any triterpenoid in *E. coli*. In order to obtain an *E. coli* chassis for production of dammarane-type tetracyclic ginsenosides precursor, we aimed to produce dammarenediol-II by reconstituting the dammarenediol-II biosynthetic pathway in *E. coli*.

Materials and methods

Strains and medium

All the strains and plasmids used in this study are listed in Supplementary Table 1.

Escherichia coli strains were cultivated in liquid lysogeny broth (LB) or terrific broth (TB) medium: LB, 10 g tryptone l^{-1}, 5 g yeast extract l^{-1} and 10 g NaCl l^{-1}; TB, 12 g tryptone l^{-1}, 24 g yeast extract l^{-1}, 4 ml glycerol l^{-1}, 17 mM KH_2PO_4 and 72 mM K_2HPO_4. Antibiotics (50 mg streptomycin l^{-1} and 50 mg kanamycin l^{-1}) were added as required.

Plasmid construction for gene expression in *E. coli*

pSQ1 was constructed from pET28a, transmembrane domain of squalene synthase (SS) (GenBank: X59959.1) from *Saccharomyces cerevisiae* which was predicted using TMHMM program (Chen et al. 2003), 26 amino acid residues *N*-terminal sequence were truncated and named ScERG9, the gene was amplified from the genomic DNA of *S. cerevisiae* W303-1a and then inserted into the *Xba*I-*Bam*HI site of pET28a. Isopentenyl diphosphate isomerase (IDI) gene (GenBank: AF119715.1) and farnesyl diphosphate synthase (IspA) gene (GenBank: KP670645.1)

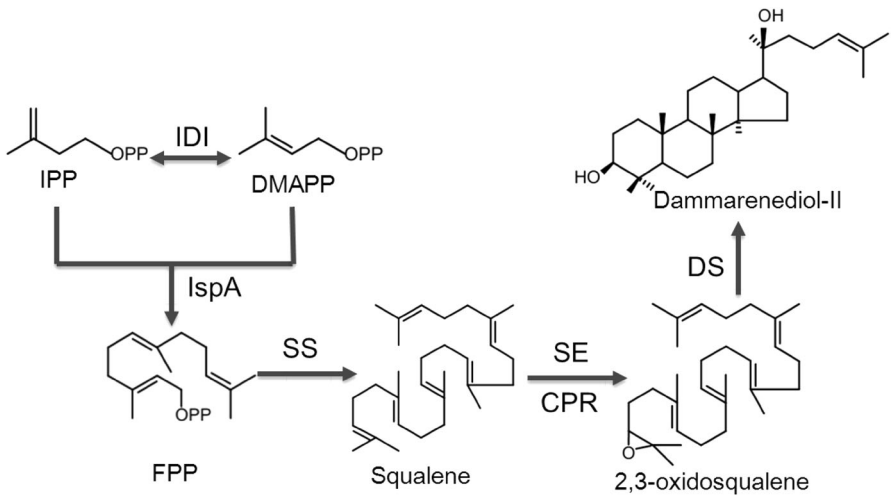

Fig. 1 Schematic diagram of the dammarenediol-II biosynthesis in *E. coli*. *IPP* isopentenyl diphosphate; *DMAPP* dimethylallyl diphosphate; *FPP* farnesyl diphosphate; *IDI* isopentenyl diphosphate isomerase; *IspA* farnesyl diphosphate synthase; *SS* squalene synthase; *SE* squalene epoxidase; *CPR* NADPH-cytochrome P450 reductase; *DS* dammarenediol-II synthase

were amplified from the genomic DNA of *E. coli* BL21 (DE3), the *idi-ispA* expression cassette was constructed by fusion PCR, and then cloned into the *Sac*I-*Hin*dIII sites of pSQ1, which yielded pSQ2.

28 and 55 amino acid residues of the *N*-terminal sequence of squalene epoxidase (SE) (GenBank: M64994.1) from *S. cerevisiae* were truncated, the two gene fragments, named *t1ScERG1* and *t2ScERG1,* were amplified from the genomic DNA of *S. cerevisiae* W303-1a (Mullner et al. 2004), respectively. Another SE (GenBank: AAU91076.1) from *Methylococcus capsulatus* was synthetized with codon usage optimization in *E. coli* which is named *McSE* (Nakano et al. 2007). The three *SEs* digested were then inserted between the *Bam*HI and *Sac*I sites of pSQ2 respectively and yielded pOSQ1-3.

45 amino acid residues of the *N*-terminal transmembrane domain of the NADPH-cytochrome P450 reductase (CPR) (GenBank: BT008426.1) from *Arabidopsis thaliana* were truncated (tAtCPR), and the gene was synthetized with codon usage optimization in *E. coli* (Han et al. 2011); 33 amino acid residues of *N*-terminal transmembrane domain of another CPR from *S. cerevisiae* (GenBank: D13788.1) were truncated (tScCPR) and the gene was amplified from the genomic DNA of *S. cerevisiae* W303-1a (Venkateswarlu et al. 1998). These two *CPR*s were digested and inserted into the *Hin*dIII and *Xho*I sites of pOSQ1-3, respectively, and six different combinations of plasmids were produced (pOSQ4-9) as shown in Supplementary Table 1 and Table 1.

The gene encoding dammarenediol-II synthase (DS) (GenBank: AB265170.1) from *Panax ginseng* was synthesized with codon usage optimized for *E. coli* and the synthetic *DS* gene was inserted into *Bam*HI and *Sac*I sites of pCDFDuet-1 which yielded pDS.

Gene induction in *E. coli* and metabolite extraction

For the induction of gene expression in *E. coli*, the transformed strains were inoculated in 5 ml liquid LB medium with appropriate antibiotics and cultured overnight at 37 °C, 200 rpm, and the 0.5 ml culture was then inoculated in 30 ml TB fermentation medium with appropriate antibiotics in 250 ml flasks and incubated at 37 °C with shaking at 200 rpm. When the OD_{600} of the culture reached 0.6–0.8, IPTG was added to 0.5 mM to induce proteins expression at 25 °C. After induction/fermentation for 48 h at 25 °C, 30 ml

Table 1 Results of the different combinations of SE and CPR

CPR	SE		
	t1ScERG1	t2ScERG1	McSE
No CPR	–	–	–
tAtCPR	–	–	+
tScCPR	–	–	+

In the strains OSQ1–OSQ9 with different combinations of SE and CPR, if 2, 3-oxidosqualene was detected, "+"; if not, "−". All experiments were performed in triplicate

culture was centrifuged at 10,000×*g* at 4 °C for 5 min. Cells were then lysed by ultrasonic extraction with 2 ml acetone three times.

The supernatant was extracted by ethyl acetate. Two ml ethyl acetate was added to 8 ml supernatant, and the mixture was agitated by vortex for 1 min. After centrifugation at 12,000×*g* for 10 min, the ethyl acetate phase was collected.

HPLC and GC–MS analysis of solvent extracts

Squalene and 2,3-oxidosqualene were analyzed by HPLC which was carried on an Elite P230II high-pressure pump system coupled with UV detection at 203 nm. Chromatographic separation was realized on Grace Apollo C18 column (4.6 mm × 250 mm, 5 μm). Squalene and 2,3-oxidosqualene (Sigma) were used as standards for quantification.

Dammarenediol-II was analyzed by GC/MS using a Agilent technologies 5975C insert xl MSD with triple-Axis Detector equipped with a HP-5 ms (30 m × 0.25 mm × 0.5 μm) GC column. Compound separation was achieved with the injector at 300 °C, and a 30 min gradient program for GC-separation starting at 80 °C for 1 min followed by heating to 300 °C at 20 °C min^{-1} and a final constant hold at 300 °C for 18 min. Mass detection was achieved with electricionization using a SIM scan mode with diagnostic ion monitored: m/z 69, m/z 109, m/z 135, m/z 363 and m/z 411 (Dai et al. 2013). A crystallized dammarenediol-II sample was used as the standard for quantification (purchased from BioBio-Pha, China).

Effect of inducing temperature, pH and IPTG concentration on dammarenediol-II production

DS1 and DS2, *E. coli* BL21 (DE3) harboring the pOSQ8 and pDS, pOSQ9 and pDS, respectively, were grown overnight in 5 ml LB medium with 50 mg streptomycin l^{-1} and 50 mg kanamycin l^{-1} at 37 °C and with shaking at 200 rpm. 0.5 ml of this culture was added to 30 ml TB fermentation medium with antibiotics in 250 ml shake-flasks and grown at 37 °C. When the OD_{600} reached 0.6–0.8, IPTG was added at 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5 mM and cultures were grown at 22, 25, 30 and 37 °C and different pH (see Fig. 3 below). Gene induction and metabolite extraction were carried out as indicated above.

Results

Reconstitution of the squalene and 2,3-oxidosqualene biosynthetic pathway and identification of 2,3-oxidosqualene produced in *E. coli*

Two *E. coli* BL21 (DE3) strains were transformed with the plasmids pSQ1 and pSQ2 respectively, which yielded strain SQ1 and strain SQ2, HPLC analysis showed that there were apparent peaks at retention time (Rt) of 33 min in SQ1and SQ2, the squalene standard had its peak at the same retention time. Then nine *E. coli* BL21 (DE3) strains were transformed with the plasmids pOSQ1 to pOSQ9 respectively and yielded the strains named OSQ1- OSQ9. The 9 strains were cultured and induced according to the methods mentioned above. The results of HPLC analysis showed that there were apparent peaks at retention time (Rt) of 15 min in OSQ8 and OSQ9, the 2,3-oxidosqualene standard had its peak at the same retention time, howerer, no apparent peak showed in OSC1-7 (Table 1; HPLC data not shown).

Reconstitution of the dammarenediol-II biosynthetic pathway and identification of dammarenediol-II produced in *E. coli*

According to the conditions of 2,3-oxidosqualene production, two strains, DS1 and DS2 harboring pOSQ8 and pDS, pOSQ9 and pDS respectively, were constructed. After culturing and induction using the methods indicated above, the metabolite extracts of DS1 and DS2 were detected and analyzed by GC–MS in positive mode (Fig. 2). It is suggested that the compound corresponding to the peak at Rt 21.4 min was dammarenediol-II and clearly showed that DS1 and DS2 both produced dammarenediol-II. During the fermentation, OSQ8 had no detectable dammarenediol-II production as a control.

Effect of inducing temperature, pH and IPTG concentration on dammarenediol-II production

A lower inducing temperature was beneficial to dammarenediol-II biosynthesis in DS1, when inducing temperature was set at 25 °C, which was about twice the production of that at 37 °C. Taking pH and IPTG concentration into account, the best condition of

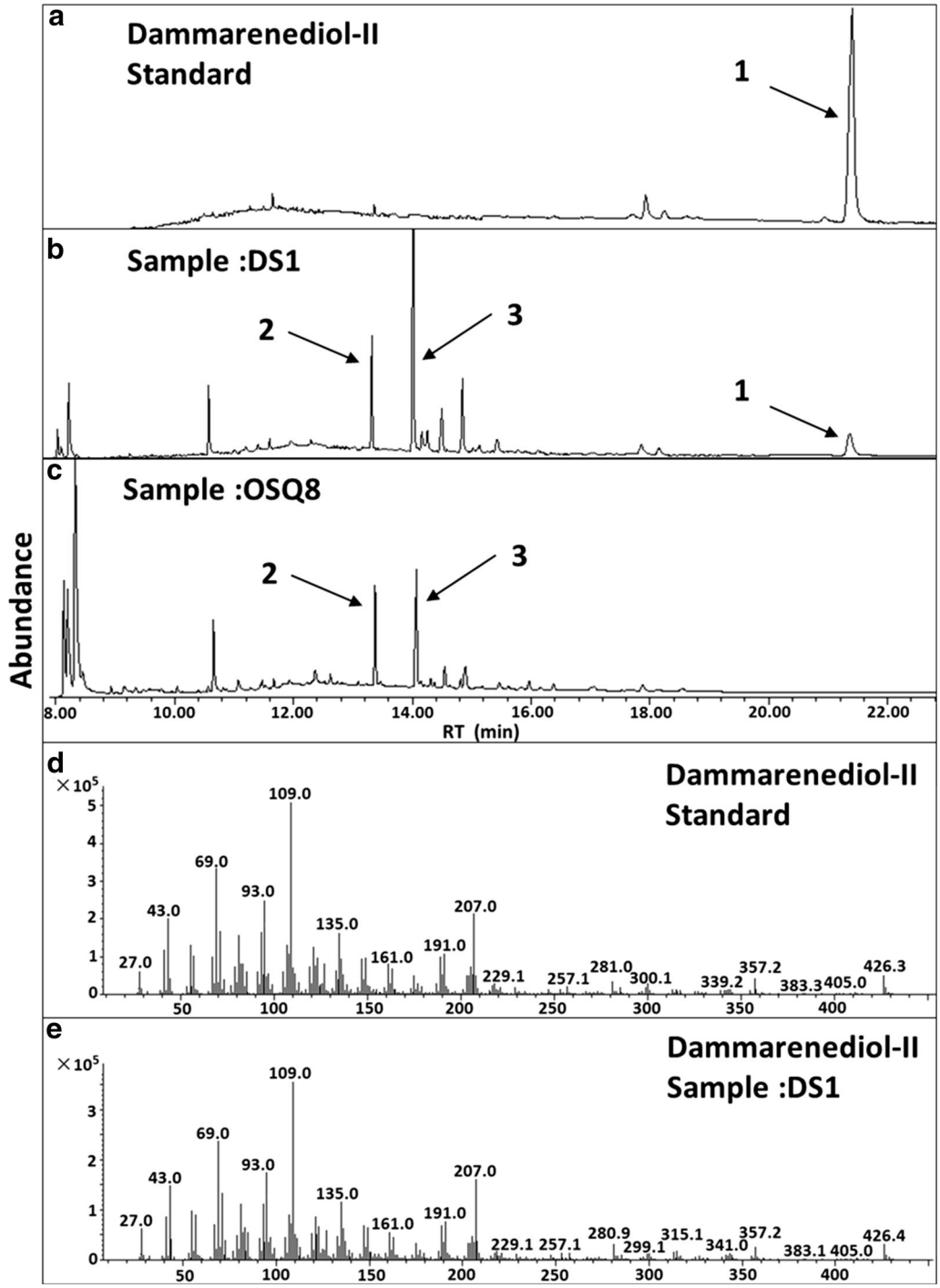

Fig. 2 Identification of fermentation products of strain DS1 and strain OSC8. These strains were cultivated in TB medium for 48 h at 25 °C. **a** GC–MS analysis of dammarenediol-II standard; **b** GC–MS analysis of cell extraction of strain DS1; **c** GC–MS analysis of cell extraction of strain OSQ8; **d** mass spectrum of dammarenediol-II standard; **e** mass spectrum of dammarenediol-II in sample DS1. (1) dammarenediol-II; (2) squalene; (3) 2,3-oxidosqualene. (mass spectra of squalene and 2,3-oxidosqualene not shown)

dammarenediol-II production was at 25 °C, pH 7.5 and 0.5 mM IPTG added to induce proteins expression. Under this condition, dammarenediol-II production reached 8.63 mg l^{-1} in DS1, and 6.47 mg l^{-1} in DS2 (Fig. 3). The supernatant extracted by ethyl acetate had no detectable dammarenediol-II, 2,3-oxidosqualene or squalene during the experiments.

Discussion

Dammarenediol-II is the precursor of many biologically active dammarane-type tetracyclic ginsenosides. Here we reconstituted dammarenediol-II biosynthetic pathway in *E. coli*, to achieve this, pSQ2 was constructed by over-expressing *idi* and *ispA* to improve the concentration of FPP which is the key precursor of terpenoids. With this improvement, there were enough precursors for the production and detectation of squalene,2,3-oxidosqualene and dammarenediol-II. For the very important step, the epoxidation of squalene to 2,3-oxidosqualene, which is carried out by the squalene epoxidase (SE), a microsomal protein that requires the participation of the flavoprotein NADPH-cytochrome P450 reductase (CPR), we constructed three plasmids pOSQ1-3 to expressed t1ScERG1, t1ScERG1 and McSE, respectively. However, none of the three strains could produce 2,3-oxidosqualene because of the lack of CPR. To solve this, six different combinations of SE and CPR were co-expressed in strains OSQ4-9. Among these six strains, only OSQ8 and OSQ9 could produce 2,3-oxidosqualene (Table 1). This is partly because neither tAtCPR nor tScCPR is an available CPR required for ScERG1 but the two CPRs can both catalyze electron transfer from NADPH to McSE. Before this, only one CPR from rat liver was used as a reductase partner of SE (Shen et al. 1989), here another two CPRs were detected to be new reductase partners of SE, in which the tAtCPR can also catalyze

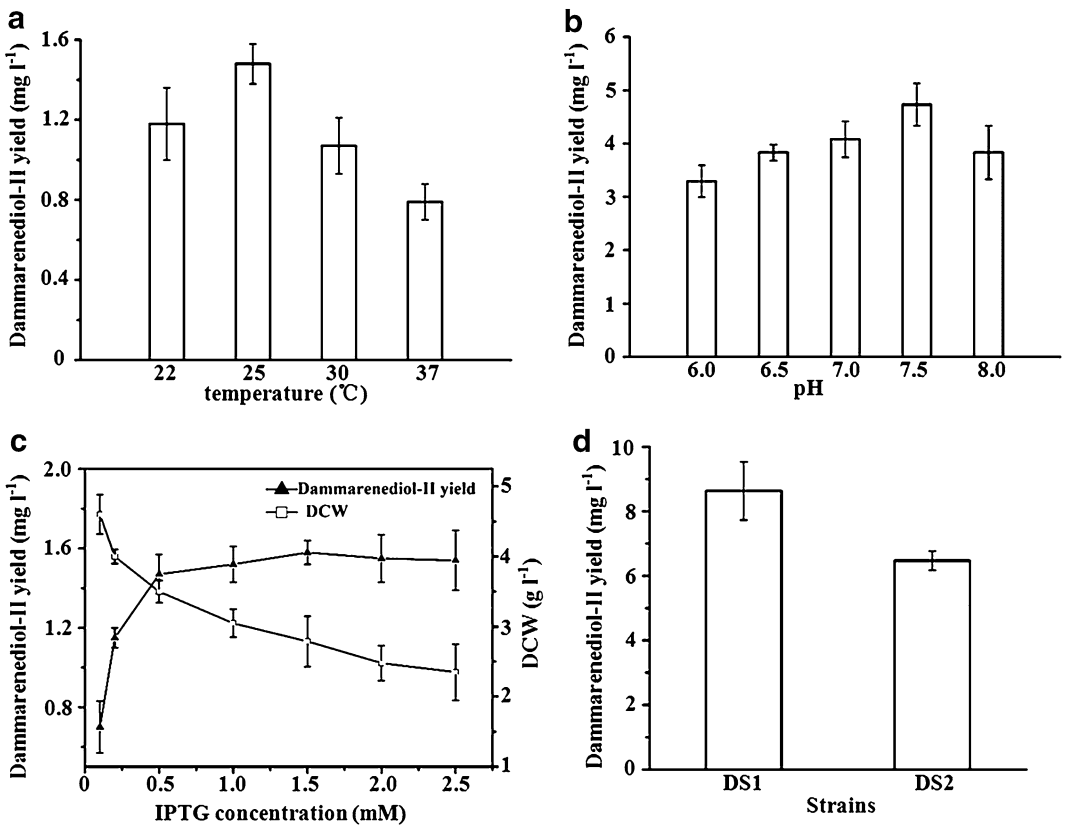

Fig. 3 Dammarenediol-II production: **a** at different temperatures; **b** at different pH; **c** under different IPTG concentrations; **d** under the best condition that at 25 °C, pH 7.5 and 0.5 mM IPTG added. *DCW* dry cell weight

electron transfer from NADPH to the cytochrome P450 s in the further downstream pathway of ginsenosides biosynthesis (Han et al. 2011).

Furthermore, we constructed pDS plasmid containing dammarenediol-II biosynthesis gene, co-transformed pDS and pOSQ8, nine plasmids into *E. coli* BL21 (DE3) and gained the DS1 and DS2 strains.The DS1 and DS2 could be induced to produce dammarenediol-II in simple TB medium, and the dammarenediol-II yield was 8.63 mg l^{-1} in 48 h fermentation. So far, two *E. coli* chassis with different SE/CPR combinations for producing triterpenoidal dammarenediol-II have been established. which could form an option for the production of triterpenoid in *E. coli*. Also, the strategy could be potentially applied in 2, 3- oxidosqualene- derived compounds production in *E. coli* when different OSCs were introduced into the system.

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Supporting information Supplementary Table 1—Plasmids and strains used in this study.

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