

Biosynthesis of a ganoderic acid in *Saccharomyces cerevisiae* by expressing a cytochrome P450 gene from *Ganoderma lucidum*[†]

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Abstract

Ganoderic acid (GA), a triterpenoid from the traditional Chinese medicinal higher fungus *Ganoderma lucidum*, possesses antitumor and other significant pharmacological activities. Owing to the notorious difficulty and immaturity in genetic manipulation of higher fungi as well as their slow growth, biosynthesis of GAs in a heterologous host is an attractive alternative for their efficient bioproduction. In this study, using *Saccharomyces cerevisiae* as a host, we did a systematic screening of 72 candidates of cytochrome P450 monooxygenase (CYP450) genes from *G. lucidum*, which are considered responsible for the GA biosynthesis from lanosterol. As a result, overexpression of a CYP450 gene *cyp5150l8* led to the production of an antitumor GA, 3-hydroxy-lanosta-8, 24-dien-26 oic acid (HLDOA) in *S. cerevisiae*, as confirmed by HPLC, LC-MS and NMR. A final titer of 14.5 mg/L of HLDOA was obtained at 120 h of the yeast fermentation. Furthermore, our *in vitro* enzymatic experiments indicate that CYP5150L8 catalyzes a three-step biotransformation of lanosterol at C-26 to synthesize HLDOA. To our knowledge, this is the first report on the heterologous biosynthesis of GAs. The results will be helpful to the GA biosynthetic pathway elucidation as well as to future optimization of heterologous cell factories for GA production. This article is protected by copyright. All rights reserved

KEYWORDS: Triterpenoid, cytochrome P450 monooxygenase (CYP450), *Saccharomyces cerevisiae*, synthetic biology, secondary metabolite biosynthesis, medicinal mushroom

1 INTRODUCTION

Ganoderma lucidum (called Ling-zhi in Chinese or Reishi in Japanese) is known as a famous traditional medicinal mushroom of immortality, and it has been used for treatment of various diseases and promotion of health for over 2000 years in China and other East Asian regions (Bishop et al., 2015; Shiao, 2003). *Ganoderma* products are nowadays consumed worldwide as a health tonic and dietary supplement with its current annual sales over US\$2.5 billion (Li et al., 2013). Ganoderic acids (GAs), a group of highly oxygenated lanostane-type triterpenoids, are well recognized as a main group of unique bioactive compounds in the medicinal mushroom (Bishop et al., 2015; Li et al., 2013; Shiao, 2003; Tang et al., 2006; Xiao and Zhong, 2016; Zhong and Xiao, 2009); and several individual GAs have been reported to possess important biological and pharmacological activities including antitumor, anti-metastasis and anti-HIV effects in both *in vitro* and *in vivo* studies (Bishop et al., 2015; Gill et al., 2016; Sato et al., 2009; Tang et al., 2006).

Despite the outstanding medicinal value of GAs, their low production has become a bottleneck for its widespread application (Xiao and Zhong, 2016; Zhong and Xiao, 2009). GAs are traditionally extracted from the fruiting bodies and spores of their native producing host *G. lucidum*, but the field cultivation of the mushroom is labor intensive and time consuming while the product quality is very difficult to guarantee (Zhong and Xiao, 2009). In a recent decade, a biotechnological approach using submerged fermentation of *G. lucidum* mycelia has been a promising alternative for GA production and many strategies have been reported to increase the GA production by fermentation (Xu et al., 2010; Xu et al., 2014; Xu and Zhong, 2012) and by metabolic engineering (Xu et al., 2012; Zhang et al., 2016). However, the genetic manipulation of mushrooms is notoriously difficult and immature while the biosynthetic pathway of GAs remains unclear, currently the metabolic engineering of the GA producing mushroom - *G. lucidum* has its limitation in enhancing the GA biosynthesis (Zhong and Xiao, 2009). Furthermore, compared to other fast growing microorganisms, the mycelial growth of mushrooms is rather slow. Those facts imply the mushroom fermentation may be practically not easy to reach a very high GA production titer and productivity.

Regarding the GA biosynthetic pathway, until late 1980s several teams around the world, especially Furuya's and Shiao's groups, had conducted an intensive research and proved the GA

biosynthesis via mevalonate pathway from glucose until lanosterol in *G. lucidum* (Hirotani et al., 1990; Shiao et al., 1989; Yeh et al., 1989) (Fig. 1); however, further biosynthetic steps from lanosterol to GA has been an unresolved puzzling question for nearly 30 years since then, which might include a series of reduction and/or oxidation of lanosterol (Shiao, 2003; Xu et al., 2010). To date, no genes involved in the biotransformation of lanosterol to GAs have been characterized, which remains to be extremely challenging.

To overcome the above-mentioned problems, synthetic biology approach may offer a favorable alternative to effective production of GAs and their analogues by using a heterologous host. Here, the brewing yeast *Saccharomyces cerevisiae* was chosen as a heterologous host for GA biosynthesis, because 1) it synthesizes lanosterol by mevalonate pathway, a proposed precursor to GAs (Xiao and Zhong, 2016), and 2) its endoplasmic reticulum and post-translational modification system may be suitable for expressing membrane bound eukaryotic cytochrome P450 monooxygenases (CYP450s) (Durairaj et al., 2015), which are considered responsible for catalyzing the conversion of lanosterol into GA (Fig. 1). In fact, heterologous biosyntheses of plant-derived natural products, such as artemisinic acid (Paddon et al., 2013), glycyrrhizin (Seki et al., 2008), miltiradiene (Dai et al., 2012), ferruginol (Guo et al., 2013), saikosapo(ge)nins (Moses et al., 2014), ginsenosides Rh2 and Rg3 (Dai et al. 2014; Wang et al. 2015), have received recent attention around the world. However, to the best of our knowledge, there are yet no reports on GA heterologous biosynthesis.

In this study, we attempt to achieve heterologous biosynthesis of GA by synthetic biology approach. At first, by directly taking an *in vivo* screening approach, we expressed various CYP candidates in a lanosterol hyper-producing strain *S. cerevisiae* YL-T3 which may facilitate the identification of CYPs involved in post-modification of lanosterol. After obtaining a functional CYP gene *cyp5150l8*, we continued the yeast fermentation followed by product purification to identify the biosynthesized GA. Furthermore, we conducted *in vitro* enzymatic experiments to better understand the CYP catalytic characteristics. We believe the work plays a leading role in elucidation of GA biosynthetic pathway, and it also provides a first example in applying synthetic biology for producing mushroom-derived secondary metabolites.

2 MATERIALS AND METHODS

2.1 Strains and Culture

Escherichia coli DH5 α (Tiangen Biotech, Beijing, China) was used as a cloning host. *G. lucidum* 260125-1 strain was cultivated, as described elsewhere (Xu and Zhong, 2012), for cDNA collection. *S. cerevisiae* YL-T3 was a BY4742 derivative strain with overexpression of hydroxy-3-methylglutaryl coenzyme A (tHMG1), farnesyl diphosphate synthase (ERG20), squalene synthase (ERG9) and squalene epoxidase (ERG1). It was constructed by integrating *tHMG1* and *lys2* into δ DNA site, and *erg20* and *erg9* into TRP site of *S. cerevisiae* BY4742, respectively. Yeast strains YL-T3 and WAT11U were grown in SC-His (containing 20 g/L of glucose, 6.7 g/L of yeast nitrogen base without amino acids (YNB), 0.7 g/L of SD-His) and YPD medium (containing 10 g/L of yeast extract, 20 g/L of beef peptone and 20 g/L of glucose), respectively (Burke et al., 2002). For other engineered yeast strains, they were grown either in SC-His, SC-His-Ura (containing 20 g/L of glucose, 6.7 g/L of YNB, 0.7 g/L of SD-His-Ura) or SC-His-Ura-Leu medium (containing 20 g/L of glucose, 6.7 g/L of YNB, 0.65 g/L of SD-His-Ura-Leu) (Dai et al., 2012) when appropriate, or in YPD medium at 30 °C and 220 rpm.

2.2 cDNA Collection

To stimulate GA biosynthesis, mycelia of *G. lucidum* were induced with Ca²⁺, and sampled after 6 days and 8 days in shaking-static cultivation (Xu and Zhong, 2012). Total RNA was extracted from those samples using Trizol reagent (Invitrogen) following the manufactures' instruction. The cDNA was synthesized with the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China).

2.3 Construction of Plasmids and Strains

A helper plasmid pRS426-HXT7p-FBA1t was constructed to yield a series of CYP expression plasmids. The HXT7p promoter, FBA1t terminator was cloned by primer HXT7p-F and HXT7p-R, FBA1t-F and FBA1t-R from the genomic DNA of *S. cerevisiae* YL-T3 (Table S1). The HXT7p promoter and FBA1t terminator were ligated into plasmid pRS426 (Christianson et al., 1992) using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China) to yield a CYP expression vector, pRS426-HXT7p-FBA1t. The ORF of CYP candidates was cloned into pRS426-HXT7p-FBA1t

to yield a series of CYP gene carrying plasmids using primer pairs described in Table S1. Another helper plasmid pRS425-TEF1p-PGK1t was constructed to yield CPR expression plasmid. The TEF1p promoter and PGK1t terminator were cloned by primer TEF1p-F and TEF1p-R, PGK1t-F and PGK1t-R from the genomic DNA of *S. cerevisiae* YL-T3 (Table S1). The TEF1p promoter and PGK1t terminator were ligated into plasmid pRS425 (Christianson et al., 1992) to yield CPR expression vector, pRS425-TEF1p-PGK1t. The ORF of *gl20687* was cloned into pRS425-TEF1p-PGK1t to yield the CPR gene carrying plasmid using primer pairs p425-GL20687-F and p425-GL20687-R (Table S1). To construct plasmid pESC-*cyp5150l8*, the ORF of *cyp5150l8* was cloned into pESC-His using primer pairs pESC-CYP5150L8-F and pESC-CYP5150L8-R (Table S1). All the primers are listed in Table S1.

The dual plasmids carrying CYP and CPR were transformed into *S. cerevisiae* YL-T3 using standard lithium acetate method (Burke et al., 2002). Void plasmids pRS426-HXT7p-FBA1t and pRS425-TEF1p-PGK1t were also transformed into YL-T3 and served as a control. The recombinant yeast cells were selected on SC-His-Ura3-Leu plates at 30 °C. The *cyp5150l8* expression plasmid and pRS426-HXT7p-FBA1t was also transformed into YL-T3, respectively. The resultant strains were selected on SC-His-Ura3 plate at 30 °C. Plasmid pESC-*cyp5150l8* and pESC-His was transformed into *S. cerevisiae* WAT11U, respectively. The resultant strains were selected on SC-His at 30 °C.

2.4 Analyses of Cell Growth, Residual Glucose, Medium Ethanol and Acetate, pH and Product Accumulation

Yeast cell growth was determined by measuring absorbance at 600 nm (OD₆₀₀) using BioPhotometer (Eppendorf, Hamburg, Germany). Glucose concentration was measured by glucose detection kit with glucose as a standard (Shifeng, Shanghai, China). The concentrations of ethanol and acetate in medium were determined by using an Agilent 1200 HPLC system (Waldbronn, Germany) equipped with a refractive index detector (RID) and an Aminex HPX-87H column (300 mm × 7.8 mm) at 65°C. These compounds were detected in 30 min by adopting 5 mM H₂SO₄ at a flow rate of 0.6 mL/min as mobile phase. The broth pH was determined with pH meter (Mettler Toledo, Greifensee, Switzerland). Products were extracted from samples using ethyl acetate. Briefly, 30-50 mL of ethyl acetate was

mixed with an equivalent volume of yeast culture broth and vortexed for 30 min. The ethyl acetate layer was collected, evaporated, re-dissolved in methanol and subjected to HPLC (Agilent, Waldbronn, Germany) analysis. Samples were assayed on a Agilent SB-C18 column (4.6 μ m, 5 mm $\times$ 250 mm). Mobile phase A contained methanol/acetic acid (100:0.1 v/v) and mobile phase B was 100% water. A linear gradient of 80% A to 100% A in 30 min and 100% A in 30-50 min at 1 mL/min was adopted.

2.5 *In Vitro* Enzymatic Reactions

The yeast YL-T3 strain expressing *cyp5150l8* (YL-T3-CYP5150L8) and that with void plasmid pRS426-HXT7p-FBA1t was cultivated respectively for microsome isolation. The strains were grown in SC-His-Ura3 at 30 °C for 24 h. Then, the seed culture was transferred to 400 mL YPD medium, cultivating at 30 °C for 48 h. The microsomal isolation was carried out on ice as follows: cells grown in YPD medium were centrifuged (2,000 g, 5 min, 4 °C) and washed by 200 mL TEK buffer (100 mM KCl, 50 mM Tris-HCl and 1 mM EDTA). After centrifugation (6,100 g, 3 min, 4 °C), the cell pellets were suspended in 50 mL extraction buffer (20 mM β -mercaptoethanol, 1% BSA, 0.6 M sorbitol, 50 mM Tris-HCl and 1 mM EDTA), broken by a Nano homogenizer (ATS Engineering Ltd., Suzhou, China) (cycle 4-5, 1000 bar, 4 °C), and centrifuged (10,000 g, 20 min, 4 °C). The corresponding supernatant was further centrifuged (100,000 g, 1 h, 4 °C). The resultant pellet was dissolved in 2-3 mL TEG buffer (30% glycerol, 50 mM Tris-HCl and 1 mM EDTA). The enzymatic assay was conducted in a total volume of 500 μ L of 90 mM Tris-HCl (pH 7.5) containing 500 μ g microsomal protein, 2 mM NADPH, and 400-600 μ M lanosterol; while 100 μ M 3-hydroxy-lanosta-8,24-dien-26-ol (HLDO) or 100 μ M 3-hydroxy-lanosta-8,24-dien-26-al (HLDA) was added in required reactions to confirm the bioconversion of HLDO or HLDA, respectively. Both *in vitro* added HLDO and HLDA were chemically synthesized by ChemPartner Co. Ltd., Shanghai, China, using lanosterol as substrate. After incubation at 30 °C and 120 rpm for 4 h, the product was extracted by ethyl acetate and subjected to HPLC and UPLC-MS analysis. In addition, the yeast WAT11U strain expressing *cyp5150l8* and void plasmid pESC-His was also cultivated respectively for microsome isolation. The strains were grown in SC-His at 30 °C for 48 h. Then, the seed culture was transferred to 400 mL YPD medium, cultivating at 30 °C for 24 h. Cells were centrifuged, washed and resuspended in 400 mL

YPL medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L galactose) at 16 °C for 48 h. The microsomal isolation was carried out as described above. The enzymatic assay was conducted in a total volume of 500 μ L of 90 mM Tris-HCl (pH 7.5) containing 500 μ g microsomal protein, 100 μ M lanosterol and 1 mM NADPH.

2.6 LC-MS and NMR Analyses

LC-MS was carried out using an ultra performance liquid chromatography (UPLC, Agilent Technologies) connected to a Q-TOF MS premier in atmospheric pressure chemical ionization (APCI) mode, equipped with a Waters BEH C18 column (1.7 μ m, 2.1 mm $\times$ 100 mm). The mobile phase 30% A (0.1% formic acid in water) and 70% B (0.1% formic acid in methanol) at 0.4 mL/min in 10 min was adopted. The NMR spectra of purified compound 1 were measured on 600 MHz Nuclear Magnetic (Avance III 600Hz, Karlsruhe, Germany) in CDCl₃ solvent at the Analytical Center of Shanghai Jiao Tong University (Minhang, Shanghai, China).

2.7 Extraction and Purification of Compound 1

The YL-T3 strain containing *cyp5150l8* expressing plasmid (YL-T3-CYP5150L8) was cultured in YPD medium for purification of compound 1. Briefly, a total of 20 L culture broth was harvested at day 5. Compound 1 was extracted by ethyl acetate as described above. Twenty mL of crude extract was pre-separated through a sephadex LH20 column (2 cm $\times$ 200 cm), and eluted with methanol. A 200 mL methanol eluted fraction was collected and further concentrated into 20 mL. Then, compound 1 from the concentrated elutes was separated by the preparative Agilent 1200 LC system (Waldbronn, Germany) equipped with a preparative ODS-BP C18 column (10 mm $\times$ 250 mm) (Dalian Elite Analytical Instruments Co. Ltd., Dalian, China). Mobile phase A contained acetonitrile/trifluoroacetic acid (100:0.1 v/v) and mobile phase B was 100% water. A linear gradient of 80% A to 90% A in 80 min at 2 mL/min was chosen. Collecting elutes from 67-70 min, 10 mg of purified compound 1 was finally obtained after freeze drying.

2.8 Yeast Fermentation

For seed culture, strains were grown in SC with appropriate amino acids dropout medium at 30 °C and 220 rpm to an OD₆₀₀ of 1-2. Then, they were inoculated into 250 mL flasks containing 50 mL of YPD or YPD40 medium (10 g/L of yeast extract, 20 g/L of beef peptone, and 40 g/L of glucose) with an initial OD₆₀₀ of 0.05. Samples were taken every day during five days of fermentation. The results represented the means $\pm$ S.D. of three independent samples.

3 RESULTS AND DISCUSSION

3.1 Selection of CYP Candidates and *In Vivo* Screening out CYP5150L8 as a Lanosterol Oxidase

A total of 219 CYP coding sequences were obtained according to the released genome information of *G. lucidum* (Chen et al., 2012). To narrow down the CYP candidates, we selected them according to the following three rationales. The first rationale is the CYPs which are physically close to lanosterol synthase (*lss*), because triterpenoid synthases and their subsequent tailoring CYPs were usually in a gene cluster (Krokida et al., 2013). Based on this rationale, two CYPs close to *lss* (*gl15671* and *cyp5359c1*) were selected (Table 1); The second rationale is the CYPs which are co-expressed with *lss* gene, and 78 CYPs including *cyp5359c1* which are co-expressed with *lss* during different developmental stages of *G. lucidum* (Chen et al., 2012), were selected (Table 1). The third rationale is the CYPs which were transcriptionally up-regulated in *G. lucidum* strains with enhanced GA production. Earlier Ren et al. (2013) reported that addition of methyl jasmonate could increase GA biosynthesis and led to the transcriptional up-regulation of three CYPs (*gl16069*, *cyp512v2* and *cyp5150l8*), among which *cyp512v2* was also co-expressed with *lss* (Chen et al., 2012). In our previous analyses of suppression subtractive hybridization of samples from two-stage shaking-static cultivation and shaking cultivation, one CYP transcript (*cyp5151A4*) was identified to exhibit a higher expression level in the higher producing two-stage process (unpublished data). Taken together, 82 CYP450s were selected as the candidates for next step investigation (Table 1).

Using the cDNA of *G. lucidum* as amplification template, except ten genes *cyp505d11*, *cyp505d13*, *cyp5139h1*, *cyp5150j7*, *cyp5150k2*, *cyp5151a4*, *cyp5351a3*, *cyp5359h1*, *cyp5359s1* and *cyp5359u3*, the other 72 CYP genes were successfully cloned into the yeast expression vector pRS426, under the control of hexose transporter promoter HXT7p. A total of five genes were predicted with the

function of cytochrome P450 reductase (CPR) in *G. lucidum*, which is responsible for the electron transfer from NADPH to CYP450 (Wang et al., 1997). One of the predicted CPRs - *gl20687* was randomly chosen and cloned into the yeast expression vector pRS425, under the control of the transcription elongation factor 1 promoter TEF1p. These dual plasmids were co-transformed into *S. cerevisiae* YL-T3 to yield engineered strains (Fig. 2).

After a systematic HPLC analysis of ethyl acetate extracts from fermentation of the engineered strains (Table 1), among these 72 candidates only the *cyp5150l8* overexpressed strain was screened out to generate a new peak in the chromatogram. As shown in Fig. 3A, peak 1 with retention time (R_t) = 24.5 min was formed in the *cyp5150l8* overexpressed strain, which was not detected in control strain with void plasmid. For other 71-CYP-overexpressed strains, e.g., the *cyp5150l6* overexpressed strain as demonstrated in Fig. 3A, neither peak 1 nor other new peaks were observed compared to the control strain (Table 1). In addition, from both the cell extract of YL-T3 overexpressed with CYP5150L8 and a predicted CPR from *G. lucidum* and that of YL-T3 solely overexpressed with CYP5150L8, similar HPLC patterns were observed (data not shown). Consequently, the CYP5150L8 solely overexpressed strain YL-T3-CYP5150L8 was adopted in later investigations.

To determine the molecular weight of the compound (peak 1 in HPLC pattern), cell extracts of the engineered yeast YL-T3-CYP5150L8 were further subjected to UPLC-MS analyses. As a result, ion chromatograms and MS spectra were obtained for peak 1 (Figs. 3B and 3C). With the detected m/z at 439.3575 (which was almost identical to the calculated molecular weight of $[C_{30}H_{48}O_3-H_2O+H]^+$, 439.3576) (Fig. 3C), the molecular formula of compound 1 from peak 1 with R_t = 5.14 min (Fig. 3B) was predicted as $C_{30}H_{48}O_3$. Compared to its substrate lanosterol ($C_{30}H_{50}O$), two oxygen atoms are added and two hydrogen atoms are missed in compound 1, implying lanosterol finally oxidized into the latter. From the above gene screening and functional identification, the CYP5150L8 is concluded as a lanosterol oxidase.

3.2 Identification of compound 1 as HLDOA and its heterologous production

Starting from 20 L YPD culture of the YL-T3-CYP5150L8 fermentation, through extraction and several purification steps, finally we obtained about 10 mg of the purified compound 1. To determine

the compound chemical structure, we did various NMR analyses. As a result, the ^{13}C -NMR and ^1H -NMR spectra data of compound 1 (Table 2, Figs. S1 and S2) were found to perfectly match with those of 3-hydroxy-lanosta-8, 24-dien-26-oic acid, a GA reported earlier (Toth et al., 1983). Integrated with the data of distortionless enhancement by polarization transfer (DEPT) spectra (Fig. S3) and 2D-NMR spectra including heteronuclear single quantum coherence (HSQC) (Fig. S4), heteronuclear multiple-bond correlation (HMBC) (Fig. S5) and homonuclear correlation spectroscopy (COSY) (Fig. S6), the chemical structure of compound 1 was precisely determined to be 3-hydroxy-lanosta-8, 24-dien-26 oic acid (HLDOA) (Fig. 1), which was called as ganoderic acid Z (GA-Z) previously (Chen et al., 2012; Xiao and Zhong, 2016). But, our careful literature perusal indicates that HLDOA is different from GA-Z (CAS registry number 294674-09-2) according to the product catalog of Chemical Abstracts Service (CAS) (Columbus, Ohio 43210, USA).

Next, we studied the fermentation kinetics of the engineered yeast YL-T3-CYP5150L8 (Fig. 4). In addition to the YPD medium containing 20 g/L of glucose (YPD), another medium YPD40 with 40 g/L of glucose was also applied in our batch fermentations in order to enhance the fermentation titer of HLDOA. In the YPD medium, no significant difference in cell growth was observed between the engineered strain and the control YL-T3 with void plasmid (data not shown). Both the cell growth and HLDOA accumulation increased rapidly after 24 h of fermentation (Figs. 4C and 4D), when the residual glucose in medium was completely consumed (Fig. 4A). Succeeding to the glucose utilization by the yeast, ethanol production and simultaneous consumption (Fig. 4A) together with acetate production and quick consumption (inset of Fig. 4A) were all observed in the fermentations with both YPD and YPD40 medium (Fig. 4A). The pH in YPD and YPD40 were around 6.0 during the first 48 h fermentation, but 1-2 pH unit differences were observed in the later stage of fermentation between 72-120 h (Fig. 4B). Although acetate was almost consumed after 72 h (inset of Fig. 4A), the pH differences may reflect the distinction in acidic metabolite secretion and consumption by the cells in YPD and YPD40 fermentation. While the cell growth nearly reached its peak around 48 h (Fig. 4C), the HLDOA production still increased until 96 h of fermentation in YPD medium, when its maximum titer was about 11.2 mg/L (Fig. 4D). No HLDOA was detectable during the fermentation of the control strain (data not shown). Compared to YPD medium, the kinetic profiles of both cell growth and

HLDOA accumulation were similar in YPD40 medium, but the cell density from 72-120 h was about 25% higher, and the final titer of HLDOA after 5-day fermentation was about 45% higher, reaching 14.5 mg/L.

De novo biosynthesis of HLDOA was achieved by overexpressing *cyp5150l8* in *S. cerevisiae*. This GA was previously reported to have interesting antitumor activities on hepatoma cells (Toth et al., 1983), and an in-depth and large-scale pharmacological investigation would be possible in future with sufficient amount of purified HLDOA available from the engineered yeast fermentation. Compared to the production titer in other reports using yeast for bioactive compound synthesis, e.g., 10.5 mg/L of ferruginol, 17.2 mg/L of protopanaxadiol, 15.9 mg/L of protopanaxatriol and 21.4 mg/L of oleanolic acid in engineered yeast (Dai et al. 2014; Guo et al. 2013), our GA titer (14.5 mg/L) is reasonable although not so high. The development of the heterologous production of the antitumor GA in the engineered yeast suggests a promising way of producing specific GAs in place of the mushroom field cultivation. The work, as the first example demonstrated, is believed to open a door to use synthetic biology approach to efficiently produce mushroom-derived triterpenoids. In future, by taking various strategies such as rational strain engineering (Paddon et al. 2013), improvement of precursor supply (Dai et al. 2012; Dai et al. 2014; Paddon et al. 2013; Soliman and Tang 2015), and other metabolic engineering optimization (Soliman and Tang 2015; Xie et al. 2014; Zhuang et al. 2017), significant increases in the GA production titer could be expected.

3.3 *In vitro* enzymatic reactions by CYP5150L8 to elucidate its catalytic steps

To confirm the lanosterol oxidase activity of CYP5150L8 as shown in the above *in vivo* screening and to understand its catalytic reaction steps, *in vitro* enzymatic assays were accordingly performed. The CYP5150L8-containing microsomes were reacted with lanosterol and NADPH, followed by UPLC-MS analysis. The *R_t* and mass spectra of compound 1 (5.14 min, [M-H₂O+H]⁺ ion at *m/z* 439.3576) and compound 2 (5.22min, [M-H₂O+H]⁺ ion at *m/z* 425.3765) were consistent with compound 1 and 2 as observed in *in vivo* experiments (Figs. 3B, 3C and 3D; Fig. 5B, 5F and 5H). Furthermore, compound 3 with a predicted molecular formula of C₃₀H₄₈O₂ was also detected as peak 3 (*R_t* = 5.55 min, Fig. 5B) with [M-H₂O+H]⁺ ion at *m/z* 423.3631 (Fig. 5J), confirming the existence of

a formyl intermediate, which was not detected in *in vivo* screening experiments. In the control assay using microsomal preparations from YL-T3 harboring void plasmid, none of compound 1, 2 and 3 was detected (Fig. 5B).

From lanosterol to HLDOA, judging their chemical structure distinction, it is reasonably inferred that the oxidation at C-26 may occur, in which the very possible conversion steps in view of organic chemistry may be three-step oxidations: step 1) The methyl group of lanosterol at C-26 oxidized into hydroxyl group; step 2) the hydroxyl into formyl group; and finally step 3) the formyl into carboxyl group (Fig. 1). To identify whether CYP5150L8 catalyzes these three-step oxidations. Microsomes from YL-T3-CYP5150L8 were incubated with two key intermediates - the hydroxyl intermediate (3-hydroxy-lanosta-8,24-dien-26-ol, HLDO) and the formyl intermediate (3-hydroxy-lanosta-8,24-dien-26-al, HLDA) (Fig. 5A, 5G and 5I). Addition of HLDO and HLDA resulted significant increase of compound 3 and compound 1 in microsomes containing CYP5150L8, respectively, while microsomes containing void plasmid were not able to catalyze the oxidation of any intermediates (Fig. 5B-D). Taken together, the *in vitro* enzymatic assay demonstrated that the CYP5150L8 was able to catalyze the above three-step oxidations.

GAs are supposed to be biosynthesized via a series of oxidation of lanosterol at different positions such as C-7, C-11, C-12, C-15, C-23, and C-26 (Shiao, 2003; Xiao and Zhong, 2016), of which the oxidation at C-26 was first discovered by this study (Fig. 1). Our results indicated that the GA - HLDOA was synthesized: from lanosterol to its hydroxyl intermediate HLDO, then from HLDO to its formyl intermediate HLDA, and finally from HLDA to HLDOA, all catalyzed by the CYP5150L8. This work has not only proved a previously supposed step from lanosterol to HLDOA (Chen et al., 2012; Xiao and Zhong, 2016), but also revealed a three-step pathway. Such a general applicable synthetic biology approach would avoid the genetic intractability of the native producer and allow researchers to discover more functional CYPs involved in GA biosynthesis. Thus, it provides a good basis for elucidating the complete biosynthesis pathway of GAs and related triterpenoids. In other words, the findings possess an indicative significance to future elucidation of biosynthesis pathways for other GAs.

In this work, the discovery of CYP5150L8 was achieved by *in vivo* screening approach (Fig. 2),

rather than *in vitro* enzymatic assay (Seki et al., 2008; Seki et al., 2011). As shown in Fig. 4D, the lanosterol oxidation product HLDOA was detectable in fermentation after 24 h, implying an easy identification of the key CYP via *in vivo* screening. In contrast, the *in vitro* enzymatic assay as we experienced was not smooth. For *cyp5150l8* overexpressed in WAT11U, a *S. cerevisiae* host for *in vitro* identification of CYPs (Urban et al. 1997), we failed to detect its *in vitro* lanosterol oxidation activity (Fig. S7A). The enzyme activity in microsomes of YL-T3-CYP5150L8 was only detected in samples from a specific fermentation time-point (Figs. S7B-D). The reasons may be a very low or instable enzyme activity or its inactivation during microsome preparation or enzymatic reaction. Compared to *in vitro* screening of CYPs, this study indicates the *in vivo* approach was quicker, more robust and straightforward. Our methodology is consistent with a recent report (Moses et al., 2014).

For CYP reactions, identifying their exact biotransformation step(s) in terpenoid biosynthesis is a highly challenging research (Guo et al., 2013). Here, from our *in vivo* and *in vitro* studies (Figs. 3-5), it is deduced that CYP5150L8 was responsible for a three-step successive C-26 oxidation of lanosterol into HLDOA (Fig. 1). In *in vitro* reactions, all reaction products - compound 1 (HLDOA), 2 (HLDO) and 3 (HLDA) were detected, but both compound 2 and 3 had a minute accumulation (Fig. 5B); in contrast, in *in vivo* experiments, compound 1 had a large accumulation as final product, compound 3 was not observed at all, and compound 2 was at a trace amount as it was very difficult to be detected in HPLC but seen in LC-MS (Fig. 3B). As compared to the control strain, a slight decrease of the substrate (lanosterol) concentration and its relatively large accumulation were always found in our *in vivo* experiments of YL-T3-CYP5150L8 (Fig. 3A), which was also true to the *in vitro* reactions (Figs. 5B and S7). The facts suggest the reaction from compound 3 (HLDA) to the final product (HLDOA) was fast, and the catalytic steps from lanosterol to compound 2 (HLDO) and from HLDO to HLDA were quite slow, while the former might be the rate-limiting step in the overall conversion reactions from lanosterol to HLDOA. This means the CYP5150L8 had a relatively high oxidase activity against HLDA, a relatively low aldehyde synthase activity against HLDO, and a very low hydroxylase activity against lanosterol. The CYP5150L8 perfectly showed its substrate promiscuity and selectivity as a CYP. Our work provides a clue to future enhancement of the HLDOA biosynthesis: engineering of CYP5150L8 for higher hydroxylase activity would be an interesting topic.

4 CONCLUSION

In this work, we have successfully characterized the first CYP - CYP5150L8 responsible for GA biosynthesis in *S. cerevisiae*. Heterologous biosynthesis of HLDOA was achieved in the engineered yeast YL-T3-CYP5150L8, with a final titer of 14.5 mg/L at 120 h of fermentation. The engineered yeast cell factory may serve as the basis for another promising way of producing specific GAs in place of mushroom field cultivation or mycelial fermentation plus extractions from those raw materials. Furthermore, the work should be helpful for discovery of more genes involved in the GA biosynthesis, which remains a fertile research area to explore.

Acknowledgments

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Additional Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Competing Financial Interests

The authors declare no competing financial interests.

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Figure Legends

Figure 1. Proposed pathway for ganoderic acid (GA) biosynthesis. Single bold arrows represent one step reaction, while triple bold arrows represent multiple step reactions. The red dashed line indicates the reaction of converting lanosterol into 3-hydroxy-lanosta-8, 24-dien-26-oic acid (HLDOA) as catalyzed by CYP5150L8. Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; HLDO, 3-hydroxy-lanosta-8,24-dien-26-ol; HLDA, 3-hydroxy-lanosta-8,24-dien-26-al; HLDOA, 3-hydroxy-lanosta-8,24-dien-26-oic acid; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IDH, isopentenyl- diphosphate isomerase; ERG20, farnesyl diphosphate synthase; ERG9, squalene synthase; ERG1, squalene epoxidase; ERG7, 2,3-oxidosqualene-lanosterol cyclase or lanosterol synthase.

Figure 2. Schematic diagram for screening CYP genes responsible for GA biosynthesis.

Figure 3. *In vivo* screening of CYP candidates. (A) HPLC analysis of extracts from fermentation of engineered *S. cerevisiae* YL-T3-CYP5150L8 (red line), other CYP overexpressed strain by taking extracts of strain YL-T3-GL24898 as an example (black line), a control strain (YL-T3 harboring a void plasmid) (blue line) at 120 h of fermentation. (B) Ion chromatograms at m/z 439, 425 and 423 m/z of the UPLC-MS analysis (total ion chromatograms) of the extracts from YL-T3-CYP5150L8 (red line) and YL-T3 harboring a void plasmid (blue line). (C) and (D) are the MS spectra of compound 1 and 2 indicated in (A) and (B).

Figure 4. Time profiles of residual glucose (circle), ethanol (square), acetate (triangle) (A), medium pH (circle) (B), cell growth (circle) (C), and accumulation of 3-hydroxy-lanosta-8, 24-dien-26-oic acid (HLDOA) (circle) (D) in shake-flask fermentation of engineered *S. cerevisiae* YL-T3-CYP5150L8 in YPD (empty) and YPD40 medium (solid). The error bars represent the standard deviation of three independent replicates. *, $p < 0.05$; **, $p < 0.01$.

Figure 5. *In vitro* enzymatic reactions by CYP5150L8. (A) Ion chromatograms of HLDOA (1), HLDO (2), HLDA (3) and lanosterol. For *in vitro* enzymatic assay, microsomes were prepared from YL-T3 containing void plasmid (control, blue line) or YL-T3-CYP5150L8 (red line). (B) - (D), 400 μ M lanosterol (B), 100 μ M HLDO (C) or 100 μ M HLDA (D) was used. Asterisks indicated substrates which were added. Microsome reaction products were analyzed by UPLC–MS in selective ion mode (m/z : 439, 425, 423 and 409). MS spectra of compound 1(F), 2 (H) and 3 (J) were consistent with that of HLDOA (E), HLDO (G) and HLDA (I), respectively.

Table 1. Screening results of CYP genes involved in ganoderic acid (GA) biosynthesis

Clan ^a	Family	Gene ID	<i>In vivo</i> screening
			results ^b
Clade2	CYP63A13	GL31768 ²	N
	CYP505D11	GL24199 ²	NA
	CYP505D12	GL19267 ²	N
	CYP505D13	GL17184 ²	NA
	CYP5136D5	GL28943 ²	N
	CYP5137A5	GL24022 ²	N
	CYP5139G1	GL31754 ²	N
	CYP5139H1	GL24426 ²	NA
	CYP5150D27	GL29831 ²	N
	CYP5150D29	GL22657 ²	N
	CYP5150J1	GL31713 ²	N
	CYP5150J10	GL24902 ²	N
	CYP5150J5	GL17382 ²	N
	CYP5150J7	GL23363 ²	NA
	CYP5150K2	GL24917 ²	NA
	CYP5150K3	GL24889 ²	N
	CYP5150L1	GL21993 ²	N
	CYP5150L3	GL22087 ²	N
	CYP5150L4	GL21992 ²	N
	CYP5150L5	GL22088 ²	N
Clade 6	CYP5150L6	GL24898 ²	N
	CYP5150L8	GL24883 ³	Y
	CYP5150L9	GL24896 ²	N
	CYP5150M1	GL24382 ²	N

	CYP5151A4	GL24198 ^{2,3}	NA
Clade 8	CYP5035K1	GL17567 ²	N
	CYP5035L2	GL17743 ²	N
	CYP5035M2	GL23374 ²	N
	CYP5035U1	GL21030 ²	N
Clade 9	CYP61A1	GL30444 ²	N
Clade 14	CYP51F1	GL26139 ²	N
Clade 15	CYP512A11	GL23303 ²	N
	CYP512A13	GL29510 ²	N
	CYP512A2	GL15605 ²	N
	CYP512A3	GL31771 ²	N
	CYP512A4	GL31772 ²	N
	CYP512A6	GL23109 ²	N
	CYP512T1	GL20660 ²	N
	CYP512U2	GL22911 ²	N
	CYP512U4	GL19231 ²	N
	CYP512U5	GL22909 ²	N
	CYP512U6	GL31761 ²	N
	CYP512U7	GL20623 ²	N
	CYP512V2	GL23338 ^{2,3}	N
	CYP512X1	GL30772 ²	N
	CYP512Y1	GL22480 ²	N
	CYP5141F2	GL18357 ²	N
	CYP5144N1	GL23174 ²	N
	CYP5140A3	GL31403 ²	N
	CYP5148B6	GL28081 ²	N
	CYP5349A3	GL21131 ²	N

Uncategorized

CYP5351A3	GL21663 ²	NA
CYP5357B1	GL31753 ²	N
CYP5359A3	GL31777 ²	N
CYP5359A5	GL15091 ²	N
CYP5359B1	GL26850 ²	N
CYP5359C1	GL31726 ^{1,2}	N
CYP5359G1	GL21057 ²	N
CYP5359H1	GL29946 ²	NA
CYP5359L1	GL20766 ²	N
CYP5359L3	GL20706 ²	N
CYP5359M1	GL31717 ²	N
CYP5359M2	GL31719 ²	N
CYP5359Q1	GL31723 ²	N
CYP5359R1	GL23851 ²	N
CYP5359S1	GL16778 ²	NA
CYP5359T1	GL23557 ²	N
CYP5359U1	GL23927 ²	N
CYP5359U2	GL23926 ²	N
CYP5359U3	GL31718 ²	NA
CYP5359W1	GL31721 ²	N
CYP5359W2	GL31722 ²	N
CYP5359Y1	GL28603 ²	N
CYP5359Y2	GL17412 ²	N
CYP5359Y5	GL31729 ²	N
CYP5359Z2	GL21090 ²	N
CYP5359Z3	GL31780 ²	N
CYP5360A1	GL22978 ²	N

CYP5365A1	GL21701 ²	N
CYP5366A1	GL30595 ²	N
-	GL15671 ¹	N
-	GL16069 ³	N

¹⁻³ Candidate CYPs were selected according to the first, second and third rationale, respectively (see text for details).

^a According to previous study, the fungal CYPs were gathered into 15 clades. (Chen et al. 2014).

^b N, no HPLC-detected new peak in cell extracts of the gene overexpressed strain as compared to the control strain; NA, not available; Y, new peak was observed in HPLC detection on cell extracts of the gene overexpressed strain as compared to the control.

Table 2. The ^{13}C -NMR and ^1H -NMR data of compound 1

C-position	^{13}C NMR(ppm)	^1H -NMR (δ ppm, J, N)
1	35.59	1.65 (m, 2H)
2	27.83	1.51 (ddd, J = 15.1, 9.0, 2.8 Hz, 2H)
3	79.02	3.15 (dd, J = 11.7, 4.4 Hz, 1H)
4	38.9	-
5	50.4	0.96 (dd, J = 12.7, 2.1 Hz, 1H)
6	18.26	-
7	28.23	1.92 (m, 2H)
8	134.33	-
9	134.43	-
10	37.03	-
11	21	1.96 (m, 2H)
12	26.5	1.93 (m, 2H)
13	44.55	-
14	49.82	-
15	30.99	1.60 (m, 2H)
16	30.83	1.08 (m, 2H)
17	50.32	1.44 (m, 1H)
18	15.78	0.60 (S, 3H)
19	18.53	0.84 (d, 6.3 Hz, 3H)
20	36.36	1.40 (d, 9.8 Hz, 1H)
21	19.16	0.91 (S, 3H)
22	34.81	-
23	25.9	(2.19m, 2.05m ,2H)

24	145.77	6.81 (t, J = 7.5Hz, 1H)
25	126.52	-
26	172.57	-
27	12	1.75 (s, 3H)
28	24.26	0.79 (s, 3H)
29	27.97	0.89 (s, 3H)
30	15.44	0.72 (s, 3H)

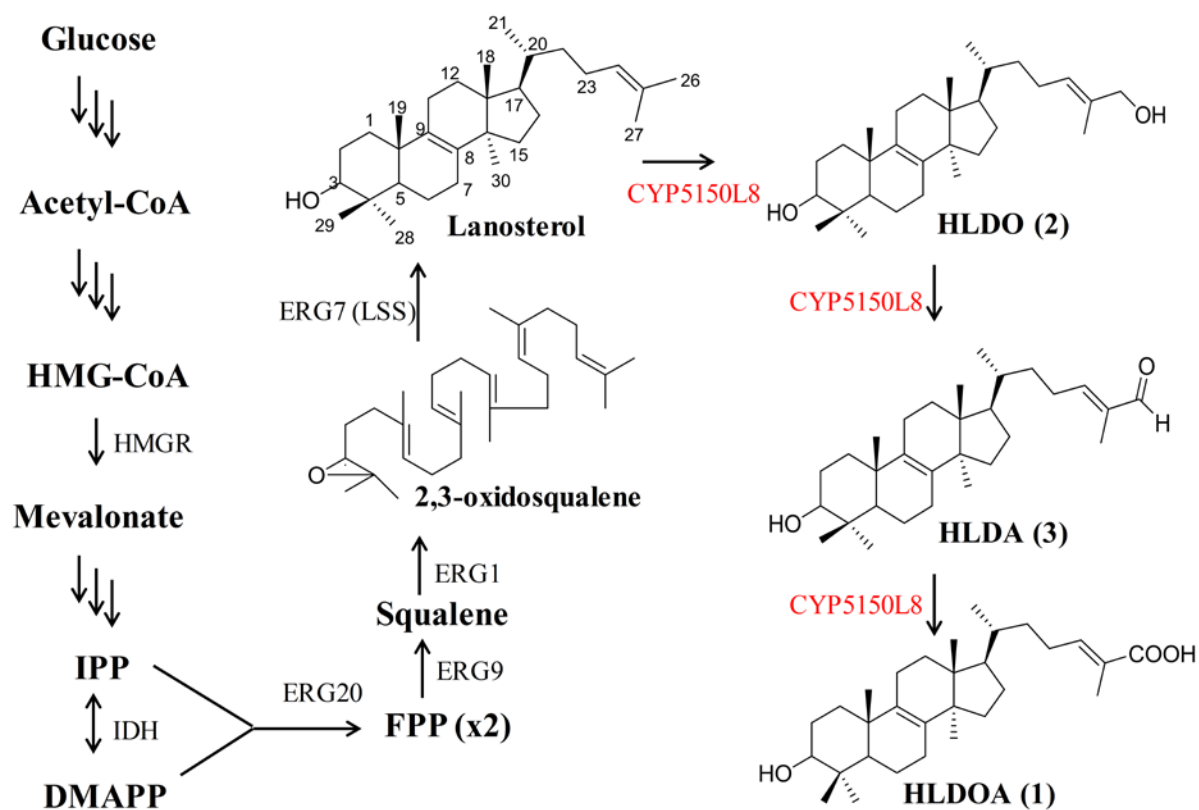


Figure 1

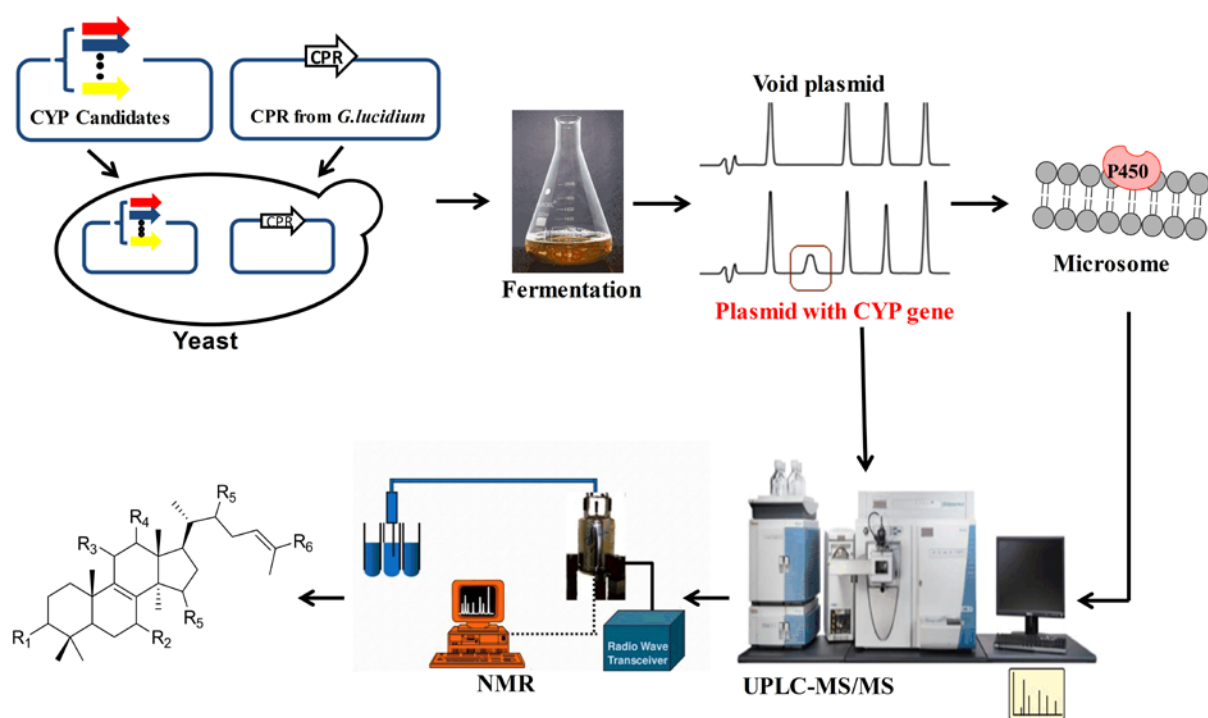


Figure 2

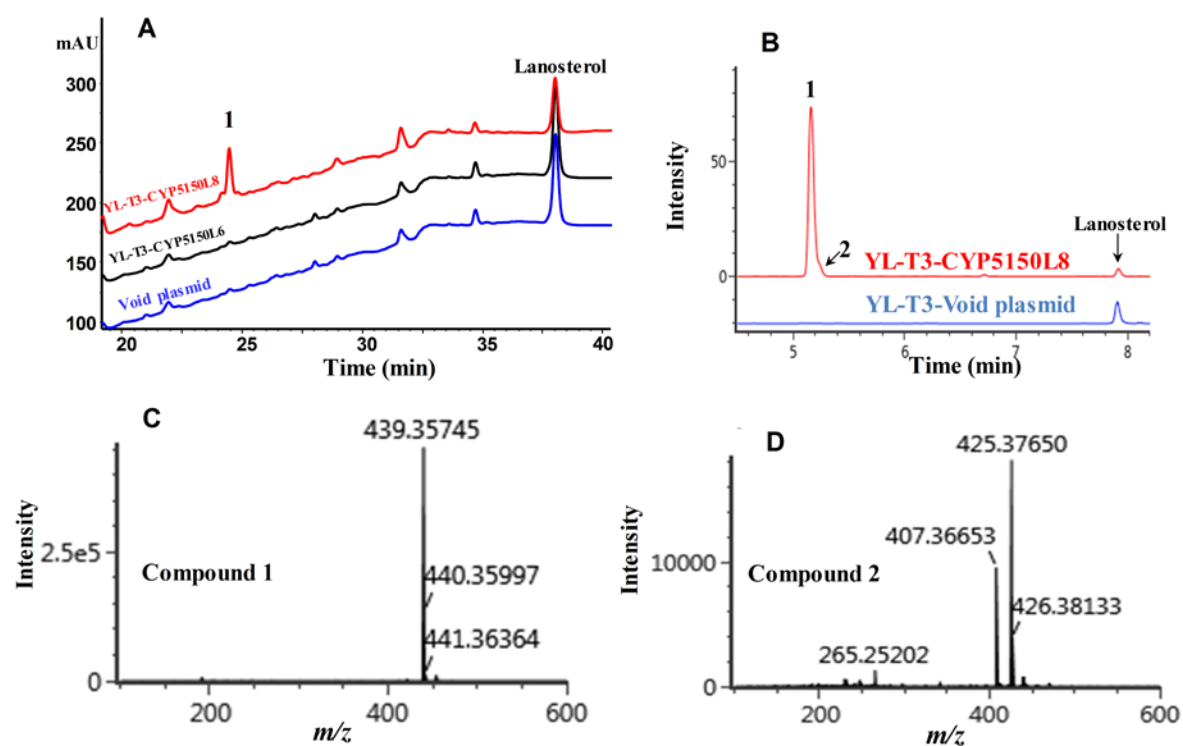


Figure 3

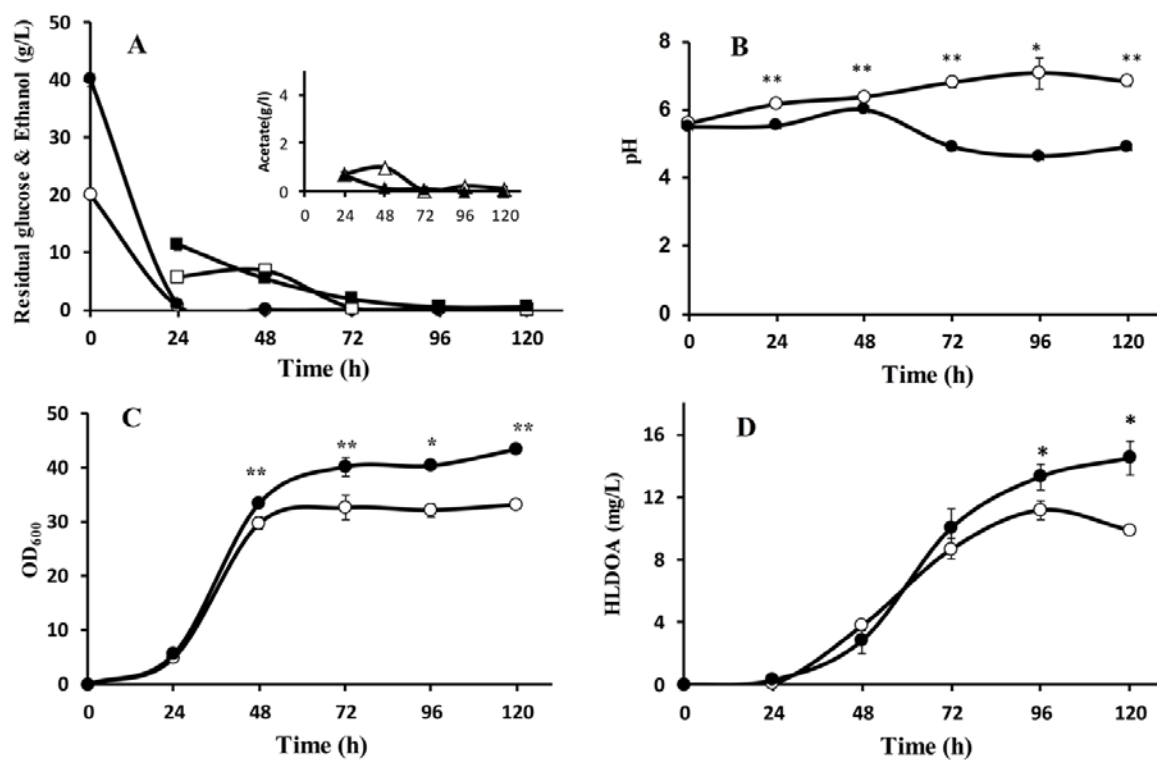


Figure 4

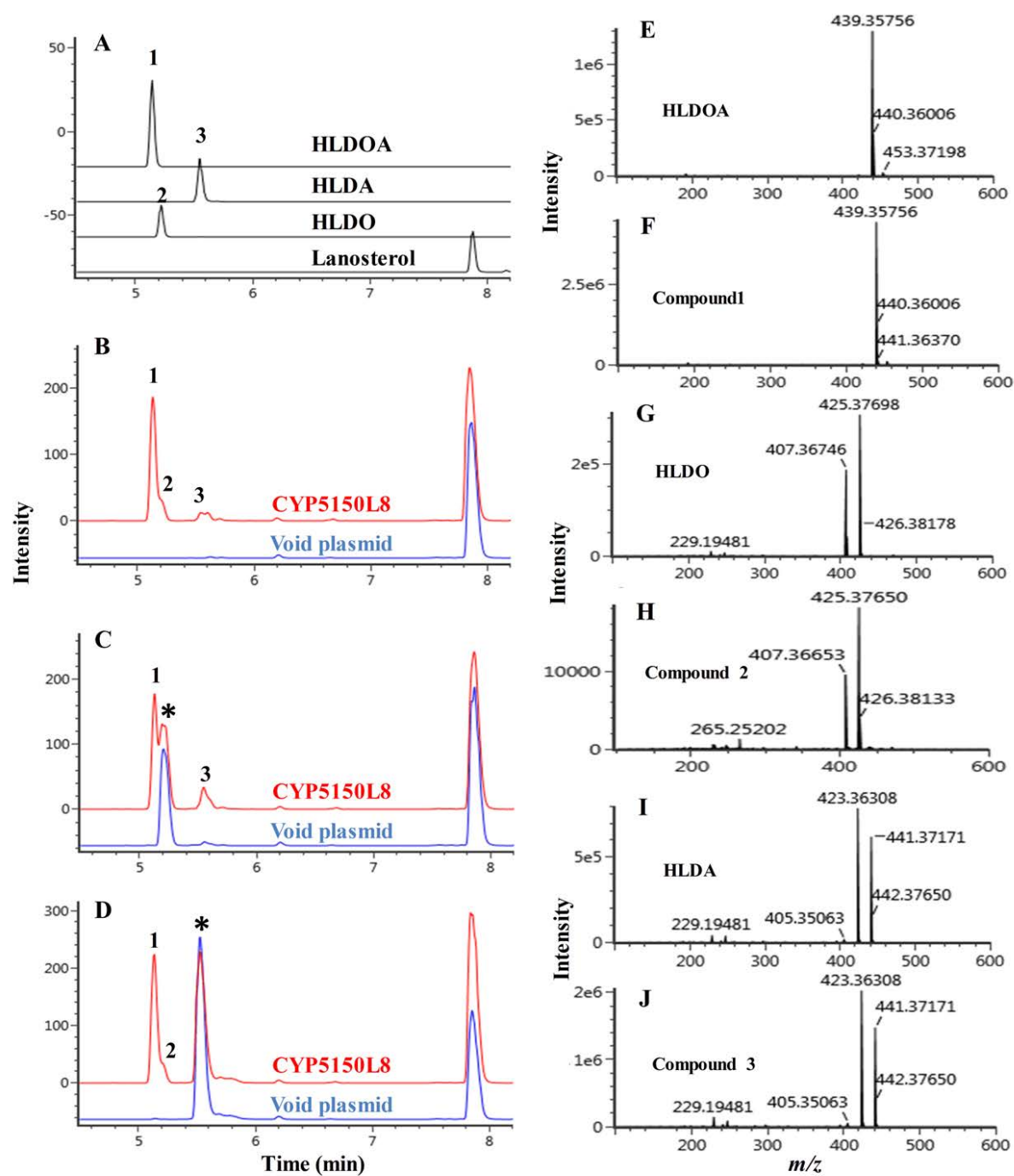


Figure 5