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# Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production

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ABSTRACT

Metabolic engineering in microbes could be used to produce large amounts of valuable metabolites that are difficult to extract from their natural sources and too expensive or complex to produce by chemical synthesis. As a step towards the production of Taxol in the yeast *Saccharomyces cerevisiae*, we introduced heterologous genes encoding biosynthetic enzymes from the early part of the taxoid biosynthetic pathway, isoprenoid pathway, as well as a regulatory factor to inhibit competitive pathways, and studied their impact on taxadiene synthesis. Expression of *Taxus chinensis* taxadiene synthase alone did not increase taxadiene levels because of insufficient levels of the universal diterpenoid precursor geranylgeranyl diphosphate. Coexpression of *T. chinensis* taxadiene synthase and geranylgeranyl diphosphate synthase failed to increase levels, probably due to steroid-based negative feedback, so we also expressed a truncated version of 3-hydroxyl-3-methylglutaryl-CoA reductase (HMG-CoA reductase) isoenzyme 1 that is not subject to feedback inhibition and a mutant regulatory protein, UPC2-1, to allow steroid uptake under aerobic conditions, resulting in a 50% increase in taxadiene. Finally, we replaced the *T. chinensis* geranylgeranyl diphosphate synthase with its counterpart from *Sulfolobus acidocaldarius*, which does not compete with steroid synthesis, and codon optimized the *T. chinensis* taxadiene synthase gene to ensure high-level expression, resulting in a 40-fold increase in taxadiene to $8.7 \pm 0.85$ mg/l as well as significant amounts of geranylgeraniol ($33.1 \pm 5.6$ mg/l), suggesting taxadiene levels could be increased even further. This is the first demonstration of such enhanced taxadiene levels in yeast and offers the prospect for Taxol production in recombinant microbes.



## 1. Introduction

Natural products such as terpenoids are often obtained by extraction from their original sources because the commercial synthesis of such highly complex structures, which often include multiple chiral centres, is too difficult and economical non viable. However, the natural sources generally produce very small amounts of these valuable target molecules, and yields vary because of environmental and epigenetic factors. Microbial terpenoids are difficult to produce if the source organism cannot be cultivated at a suitable scale. Recombinant microbial systems can be used to circumvent these hurdles (Chang and Keasling, 2006).

Today, there are more than 50,000 known terpenoids, many of which are synthesized by plants (Conolly and Hill 1991). Isoprenoids in plants often carry out specialized functions, e.g. in communication, defence and pollination attraction. Taxol (paclitaxel) is one of the better-known plant-derived terpenoids because of its pharmacological properties. Taxol is a highly functionalized diterpenoid, originally isolated from the bark of the pacific yew tree (*Taxus brevifolia* Nutt.) and now derived by semisynthesis for other advanced taxoids (obtained from various *Taxus* spp.) or produced in cultured plant cells (Suffness and Wall, 1995; Kingston, 2007). The various *Taxus* species produce arrays of chemically similar taxoid structures, with more than 350 different known taxoid structures (Baloglu and Kingston, 1999). The extraction of specific taxoids from plant material can therefore be hampered by low yields of the desired compound and the complex background of chemically closely related structures. Taxol co-purifies with many other taxoids and represents only a minor fraction of the total taxoid extract, making laborious and costly separation processes necessary. The biosynthesis of Taxol involves at least 19 enzyme-catalysed steps starting from the universal diterpenoid precursor geranylgeranyl diphosphate. Several of these biosynthetic steps are now characterized and the corresponding genes have been cloned (Jennewein et al., 2004b; Walker and Croteau, 2001). The pathway committing step

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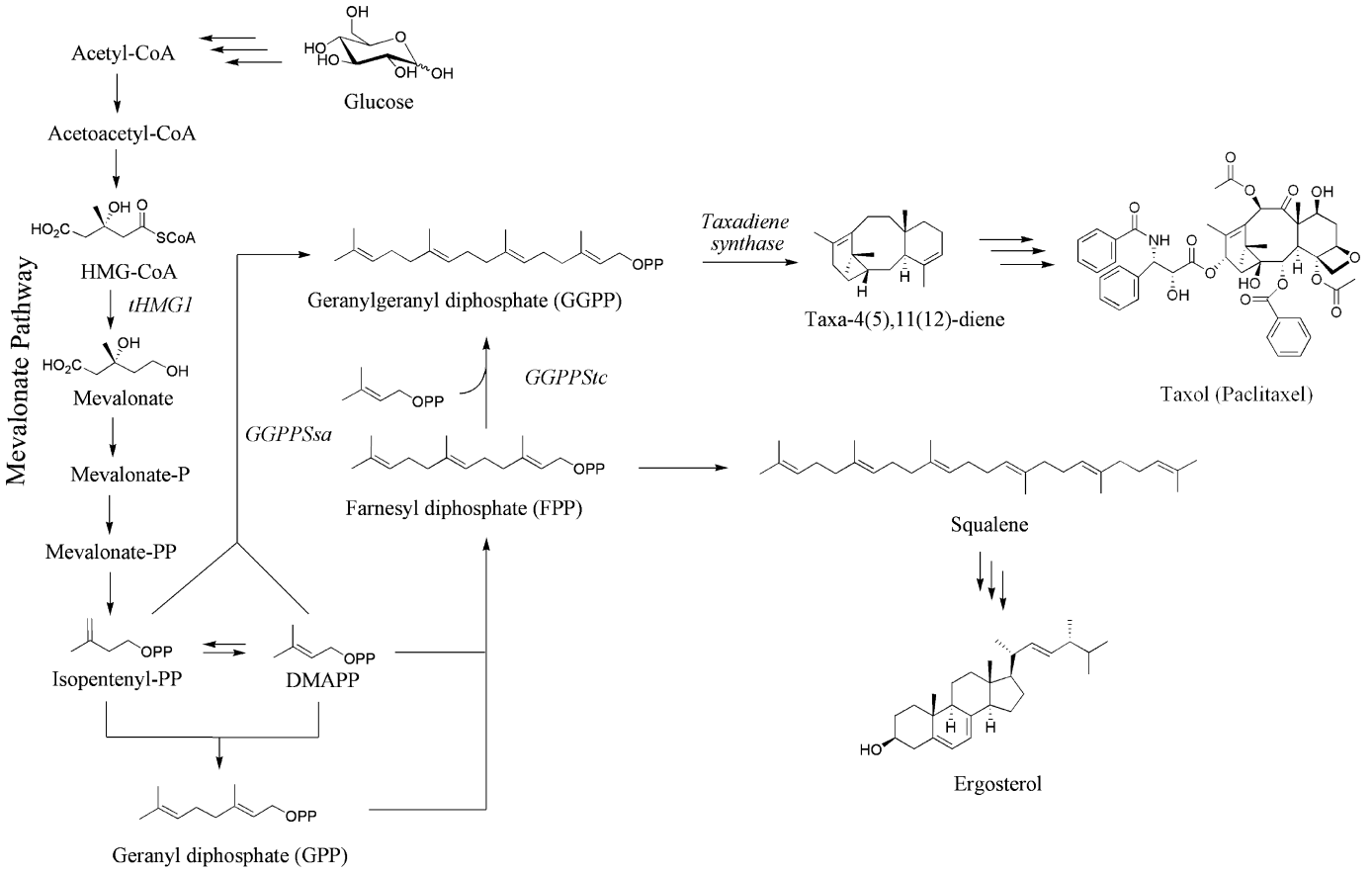


**Fig. 1.** Schematic representation of the engineered taxadiene biosynthetic pathway in competition with *Saccharomyces cerevisiae* primary metabolism. Taxadiene production in *S. cerevisiae* involved the expression of a truncated version of the yeast HMG-CoA reductase (*tHMG1*), geranylgeranyl diphosphate synthase obtained either form *Taxus chinensis* (*GGPPStc*) or *Sulfolobus acidocaldarius* (*GGPPSsa*) in combination with *Taxus chinensis* taxadiene synthase. This potentially provides the bases for further conversion into the anticancer drug Taxol.

is catalyzed by taxadiene synthase, yielding the tricyclic diterpenoid pentamethyl [9.3.1.0][3,8] tricyclopentadecane, commonly referred to as taxadiene (Koepp et al., 1995; Wildung and Croteau, 1996). Taxadiene is then oxygenated by several cytochrome P450-dependent monooxygenase-catalysed oxygenation reactions to generate Taxol, which contains eight oxygens (Hefner et al., 1996; Schoendorf et al., 2001; Wheeler et al., 2001; Jennewein et al., 2004a) (Fig. 1). Certain endophytic fungi of *Taxus* spp. can also synthesize Taxol, although the yields are much lower than in *Taxus* spp. themselves (Stierle et al., 1993). Microbial production of taxoids would offer an economic alternative to extraction from plant biomass. Also, since taxoid biosynthesis in plants is an anastomosing pathway, metabolic engineering would offer the opportunity to establish a linear pathway focusing on specific, desired taxoid products. Initial attempts to engineer Taxol biosynthesis in *Saccharomyces cerevisiae* showed that yeast primary metabolism provides very limited amounts of geranylgeranyl diphosphate, so no taxadiene was produced (DeJong et al., 2005). Only the introduction of the *Taxus canadensis* geranylgeranyl diphosphate synthase gene yielded detectable amounts of taxadiene of approximately 1 mg/l.

## 2. Material and methods

### 2.1. Yeast and bacterial strains and vectors

Yeast strain CEN.PK2-1C (MATa; ura3-52; trp1-289; leu2-3_112; his3 Δ1; MAL2-8$^c$; SUC2) was obtained from EUROSCARF (EUROpean Saccharomyces Cerevisiae Archive for Functional analysis), Universität Frankfurt (Frankfurt, Germany) (van Dijken et al., 2000). *Sulfolobus acidocaldarius* (DSMZ 639) was obtained from Deutsche Sammlung Mikroorganismen und Zellkulturen (Braunschweig). *Escherichia coli* strains TOP10, Mach1$^{TM}$-T1$^R$ and DH5α were purchased from Invitrogen and used for general cloning procedures. The Gateway compatible plasmid pDONR221 was obtained from Invitrogen, the yeast expression plasmids pVV200 and pVV214 (van Mullen et al., 2003) from EUROSCARF and the pRS313 and pRS315 vectors (Sikorski and Hiether, 1989) from American Type and Culture Collection (ATCC), through LGC Promochem (Wesel, Germany). The taxadiene synthase gene was codon optimized for improved yeast expression by Geneart (Nürnberg, Germany).

### 2.2. Construction of yeast expression vectors

For functional expression in *S. cerevisiae*, genes were amplified by PCR with gene-specific forward primers containing Gateway cloning compatible *att*B sites and a yeast consensus sequence for proper initiation of translation. The corresponding reverse primer contained gene-specific sequences, the nucleotides for a C-terminal $His_4$-tag and a 5′ terminal *att*B site. Fragments were purified using a PCR purification kit and cloned into the pDONR221 vector by Gateway cloning according the supplier's protocols. The Gateway BP Clonase$^{TM}$ products were then introduced into chemically competent *E. coli* cells and from individual clones, obtained by selection on LB-agar plates containing the

antibiotic kanamycin (50 μg/ml), the resulting pEntry clones were isolated and sequenced. Correct pEntry clones were then used to introduce each transgene into the yeast expression vectors pVV200 or pVV214. *Taxus chinensis* and *S. acidocaldarius* geranylgeranyl diphosphate synthase were cloned into pVV200, and *T. chinensis* and the codon optimized taxadiene synthase were cloned into pVV214 vector. *S. cerevisiae* tHMG-CoA reductase (truncated 3-hydroxy-3-methylglutaryl-CoA reductase) (*thmgr*) and the transcription factor UPC2.1 (*upc2.1*) were first introduced into the yeast expression vector pVV200. The pVV200-*thmgr* vector construct was then used as template for a PCR reaction to amplify the *thmgr* gene together with its *PGK1* promoter and cytochrome *C* terminator sequence using forward primer 5′-GTA ATA CGA CTC ACT ATA GGG CGA ATT GGC GGC CGC CCG ACT CTT TTC TTC TAA CCA AGG-3′ incorporating a NotI site, and reverse primer 5′-CGT GCC GCG GCA AAG CTT GCA AAT TAA AGC CTT CG-3′ incorporating a SacII site. Purified DNA fragments were digested with NotI and SacII endonucleases and ligated into pRS315 digested with the same enzymes. The pVV200::*UPC2.1* vector construct was used as template for a PCR amplification with forward primer 5′-GTA ATA CGA CTC ACT ATA GGG CGA ATT GGC GGC CGC CCG ACT CTT TTC TTC TAA CCA AGG-3′ and reverse primer 5′-CTA GAG AGC TCC ACC GCG GTG GCG GCC GCA AAG CTT GCA AAT TAA AGC CTT CG-3′. The resulting DNA fragment, comprising the PGK promoter, UPC2.1 and CYC1 terminator, was purified and digested using NotI and SacII, and ligated after purification into pRS313 linearized with the same enzymes. In each case, the correct insertion of transgene was confirmed by vector insert sequencing.

### 2.3. Yeast transformation and cultivation

Yeast was transformed using the lithium acetate method (Amberg et al., 2005) with selection on SC minimal medium agar plates (0.17% w/v yeast nitrogen base without amino acids, 0.5% w/v ammonium sulfate, 2% w/v glucose, 40 mg/l histidine, 40 mg/l tryptophane, 40 mg/l leucine, 20 mg/l uracil, 1.6% w/v agar) omitting the following components for selection of the individual plasmids: pVV200 (tryptophane), pVV214 (uracil), pRS313 (histidine) and pRS315 (leucine). For isoprenoid production, yeast was cultivated in SC minimal medium until an OD of 1 was reached, and then collected by centrifugation and resuspended in a 10-fold greater volume of buffered YPD medium (1% w/v yeast extract, 2% w/v tryptone, 2% w/v glucose, 100 mM Tris–Cl, 1 mM $MgCl_2$, pH 8) containing 0.5% w/v RPC18 silica gel for product absorption. Yeast cultures were harvested for isoprenoid analysis after 48 h. Heterologous expression of the *T. chinensis*, *S. acidocaldarius* or synthetic gene constructs showed no adverse effects in any of the recombinant yeast strains, and 21.6±2.5 g/l biomass was obtained. Yeast cultivation was carried out in 500 ml baffled Erlenmeyer flasks containing 100 ml of medium. All cultures were shaken at 160 rpm using a EB VKS-75 controlled shaker at 28 °C. All fermentation experiments were performed in triplicates and errors reported as standard deviations.

### 2.4. Terpenoid extraction and analysis

Biomass and RP18 silica gel from fermentation cultures was separated from the culture medium via filtration and then lyophilized. This was followed either by direct extraction with pentane containing 4-4′-di-*tert*-butyl-diphenyl as an internal standard or treatment for analysis of free geranylgeranyl diphosphate content. The latter involved mixing with 0.5 M potassium hydroxide solution at 60 °C for 30 min, neutralization and extraction as above. Pentane extracts were then analysed by GC/MS using a Shimadzu QP2010S quadrupole mass spectrometer equipped with a Rxi™-5 ms (0.25 mm ID, 30 m length) GC column (Restek, Bad Homburg) using a slit/slitless injector. Compound separation was achieved with an injector temperature of 250 °C, and a 22.5 min temperature gradient program for GC-separation starting at 200 °C for 3 min followed by heating the column at 4 °C/min to 270 °C and a final constant hold at 270 °C for 2 min. Mass detection was achieved with electric ionization at 1 keV using a SIM/Scan mode with diagnostic ion monitored: *m*/*z* 272 [$P^+$], 122 (highly characteristic C-ring fragment (Koepp et al., 1995; Lin et al., 1996), 107 (*m*/*z* 122 base peak –$CH_3$). A taxadiene sample obtained from a bioorganic synthesis reaction was used as the standard for quantification.

## 3. Results and discussion

In order for *S. cerevisiae* to produce Taxol, its primary metabolism must supply the necessary precursor, geranylgeranyl diphosphate. Although *S. cerevisiae* produces large amounts of farnesyl diphosphate for steroid biosynthesis, only small amounts of geranylgeranyl diphosphate are available, i.e. 122±3 μg/l in the wild-type strain CEN.PK2-1C that we used in our experiments (van Dijken et al., 2000) (Fig. 1). Therefore, when we expressed a transgene encoding the pseudomature form of the *T. chinensis* taxadiene synthase, which lacks the N-terminal, 74-residue plastid-targeting sequence (Williams et al., 2000), there was still insufficient geranylgeranyl diphosphate to support a heterologous taxoid biosynthetic pathway. The gene was controlled by the strong, constitutive phosphate glycerol kinase (*PGK*) promoter (Table 1), which is compatible with yeast fermentation using glucose as the carbon source instead of switching to galactose for transgene expression as required for yeast *GAL* promoters (Rosenberg et al., 1990).

In previous experiments, limited production of taxadiene could be achieved through the galactose-inducible expression of *T. canadensis* geranylgeranyl diphosphate synthase (DeJong et al., 2005). Therefore, we transformed *S. cerevisiae* with the pseudomature form of *T. chinensis* taxadiene synthase as discussed above together with the pseudomature form of *T. chinensis* geranylgeranyl diphosphate synthase (Hefner et al., 1998) controlled by the same *PGK* promoter. This resulted in the production of 204±1 μg/l taxadiene.

**Table 1**
Strains of *Saccharomyces cerevisiae* created in this study

| Strain no. | Strain containing the following expression vectors |
|---|---|
| CEN1 | CEN.PK2-1C |
| CEN2 | CEN.PK2-1C; pVV214::*T.c.-tds* |
| CEN3 | CEN.PK2-1C; pVV214::*T.c.-tds*; pVV200::*T.c.-ggpps* |
| CEN4 | CEN.PK2-1C; pVV214::*T.c.-tds*; pVV200::*S.a.-ggpps* |
| CEN5 | CEN.PK2-1C; pVV214::*T.c.-tds*; pVV200::*T.c.-ggpps*; pRS315::*S.c.-thmgr1* |
| CEN6 | CEN.PK2-1C; pVV214::*T.c.-tds*; pVV200::*S.a.-ggpps*; pRS315::*S.c.-thmgr1* |
| CEN7 | CEN.PK2-1C; pVV214::*T.c.-tds*; pVV200::*T.c.-ggpps*; pRS315::*S.c.-thmgr1*; pRS313::*S.c.-upc2.1* |
| CEN8 | CEN.PK2-1C; pVV214::*T.c.-tds*; pVV200::*S.a.-ggpps*; pRS315::*S.c.-thmgr1*; pRS313::*S.c.-upc2.1* |
| CEN9 | CEN.PK2-1C; pVV214::*Syn-tds*; pVV200::*T.c.-ggpps*; pRS315::*S.c.-thmgr1*; pRS313::*S.c.-upc2.1* |
| CEN10 | CEN.PK2-1C; pVV214::*Syn-tds*; pVV200::*S.a.-ggpps*; pRS315::*S.c.-thmgr1*; pRS313::*S.c.-upc2.1* |

*Abbreviations*: *T.c.-tds*, *Taxus chinensis* taxadiene synthase; *Syn-tds*, synthetic codon optimized taxadiene synthase; *T.c.-ggpps*, *Taxus chinensis* geranylgeranyl diphosphate synthase; *S.c.-thmgr1*, *Saccharomyces cerevisiae* truncated HMG-CoA reductase 1; *S.c.-upc2.1*, *Saccharomyces cerevisiae upc2.1*; *S.a.-ggpps*, *Sulfolobus acidocaldarious* geranylgeranyl diphosphate synthase.

Isoprenoid building blocks are provided in yeast exclusively via the mevalonate (MVA) pathway, and are mostly used for steroid biosynthesis, although other non-sterol products such as ubiquinone, dolicol, haem A and farnesylated proteins (e.g. yeast mating factors) are also synthesized. The yeast MVA pathway is subject to complex feedback regulation, with HMG-CoA reductase the principal regulatory target (Dimster-Denk et al., 1994). HMG-CoA reductase catalyses the conversion of HMG-CoA to mevalonate, and is regulated at the transcriptional and posttranscriptional levels. Removal of the N-terminal regulatory domain from yeast HMG-CoA reductase isoenzyme 1 (*HMG1*) prevented steroid-based negative feedback of the MVA pathway, and thus increased the supply of isopentenyl diphosphate for other isoprenoid pathways (Donald et al., 1997). The use of this truncated HMG-CoA reductase allows increased production of artemisinic acid in recombinant *S. cerevisiae* (Ro et al., 2006).

Expression of the truncated version of yeast HMG-CoA reductase (*tHMG1*) in combination with *T. chinensis* geranylgeranyl diphosphate synthase and *T. chinensis* taxadiene synthase (all controlled by the *PGK* promoter) resulted in a significant increase in taxadiene production to $306 \pm 3$ μg/l, which is 50% higher than achieved with the two *Taxus* genes (Fig. 2).

To increase the metabolic flux towards taxadiene synthesis, it is also possible to reduce the flux towards the steroid synthesis pathway. Under aerobic growth conditions, yeast does not utilize steroids provided in the culture medium, an effect known as aerobic sterol exclusion (Andreason and Stier, 1953; Lorenz and Parks, 1991). In contrast, under anaerobic conditions it is necessary to supply exogenous steroids because yeast cannot perform vital cytochrome P450-mediated monooxygenation reactions in the absence of oxygen. A key regulator of yeast steroid uptake is the transcription factor UPC2 (Vik and Rine, 2001). The mutant allele *upc2.1* encodes a protein variant that allows sterol uptake under aerobic conditions (Lewis et al., 1988; Davies et al., 2005), reflecting a single transition from guanine to adenine, which converts the C-terminal glycine residue to an aspartate residue (Crowley et al., 1998). Sterol production is energetically costly for the cell and interferes with taxoid biosynthesis by competing for the same isoprenoid precursors. Therefore, by providing means for the cell to take up exogenous steroids from the environment, we aimed to inhibit sterol production and increase the flux towards taxoid biosynthesis. We generated the *upc2.1* allele through PCR-mediated site-directed mutagenesis of the wild type *UPC2* gene and expressed this in combination with the three genes listed above (strain CEN7), resulting in taxadiene levels of $306 \pm 1$ μg/l, identical to results in the absence of *upc2*-1 (Fig. 2). Also the levels of geranylgeraniol were only marginally higher in CEN7 ($283 \pm 32$ μg/l) than CEN5 ($253 \pm 34$ μg/l).

Like other plant-derived geranylgeranyl diphosphate synthases, *T. chinensis* geranylgeranyl diphosphate synthase relies on both farnesyl diphosphate and isopentenyl diphosphate as substrates for the synthesis of geranylgeranyl diphosphate (Hefner et al., 1998). Therefore, *T. chinensis* geranylgeranyl diphosphate synthase competes with squalene synthase for farnesyl diphosphate, which is used in sterol biosynthesis. In addition, western blots of crude protein extracts from the recombinant yeast strains revealed poor expression of *T. chinensis* geranylgeranyl diphosphate synthase (results not shown), probably reflecting the unfavourable codon usage profile.

We identified a geranylgeranyl diphosphate synthease in *S. acidocaldarius* with the ability to synthesize geranylgeranyl diphosphate through sequential addition of dimethylallyl diphosphate (DMAPP) building blocks (Ohnuma et al., 1994) thus avoiding competition for farnesyl diphosphate (Fig. 1). We cloned the gene from *S. acidocaldarius* genomic DNA (strain DSMZ 639) and introduced it into our recombinant yeast strain under the *PGK* promoter as before. The heterologous expression of *S. acidocaldarius* geranylgeranyl diphosphate synthase in addition to *T. chinensis* taxadiene synthase, tHMG-CoA reductase and *upc2-1* (strain CEN8) resulted in only marginal increases in taxadiene production ($320 \pm 3$ μg/l), but significant increases in geranylgeraniol ($27.6 \pm 3.4$ mg/l), almost 100-fold more than in strain CEN7 (expressing the *T. chinensis* geranylgeranyl diphosphate synthase). The *tHMGR1* gene also had a strong impact on geranylgeraniol production, resulting in a nearly four-fold increase between strain CEN4, which lacked the gene ($6.9 \pm 2.1$ mg/l) and strain CEN6 which expressed it ($27.4 \pm 3.4$ mg/l). Thus, taxadiene synthase activity represents the limiting factor in the production of taxadiene in yeast strains with improved provision of geranylgeranyl diphosphate as shown in Fig. 3.

Northern and western blots showed that the *T. chinensis* taxadiene synthase gene was poorly translated in yeast due to the possession of several rare arginine codons. To improve taxadiene synthase expression levels, the amino acid sequence was codon-optimized for *S. cerevisiae* and two further yeast strains were created containing codon-optimized *T. chinensis* taxadiene synthase, truncated HMG-CoA reducatase, the *UPC2-1* transcription factor gene and either the *T. chinensis* geranylgeranyl diphosphate synthase (CEN9) or its *S. acidocaldarius* equivalent (CEN10). While CEN9 had a taxadiene content of $228 \pm 15$ μg/l,

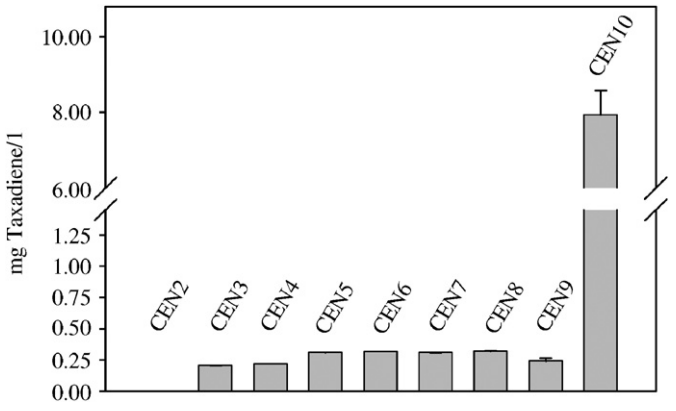


**Fig. 2.** Production of taxa-4(5),11(12)-diene by recombinant *S. cerevisiae* strains as listed in Table 1. *S. cerevisiae* cultures were cultivated using glucose as carbon source over 48 h, freeze dried, extracted with pentane and analyzed by GC/MS for taxadiene content. Data are represented as mean total production ± s.d. from three independent fermentations.

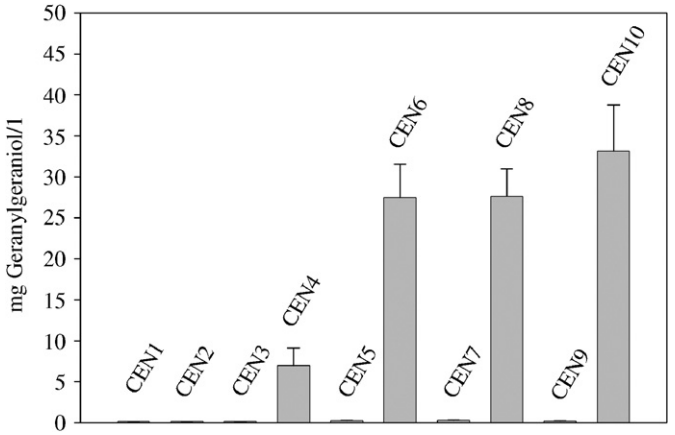


**Fig. 3.** Production of geranylgeraniol by the recombinant *S. cerevisiae* strains listed in Table 1. Geranylgeraniol content of the taxadiene fermentation cultures determined by GC/MS. Data are represented as mean total production ± s.d. from three independent fermentations.

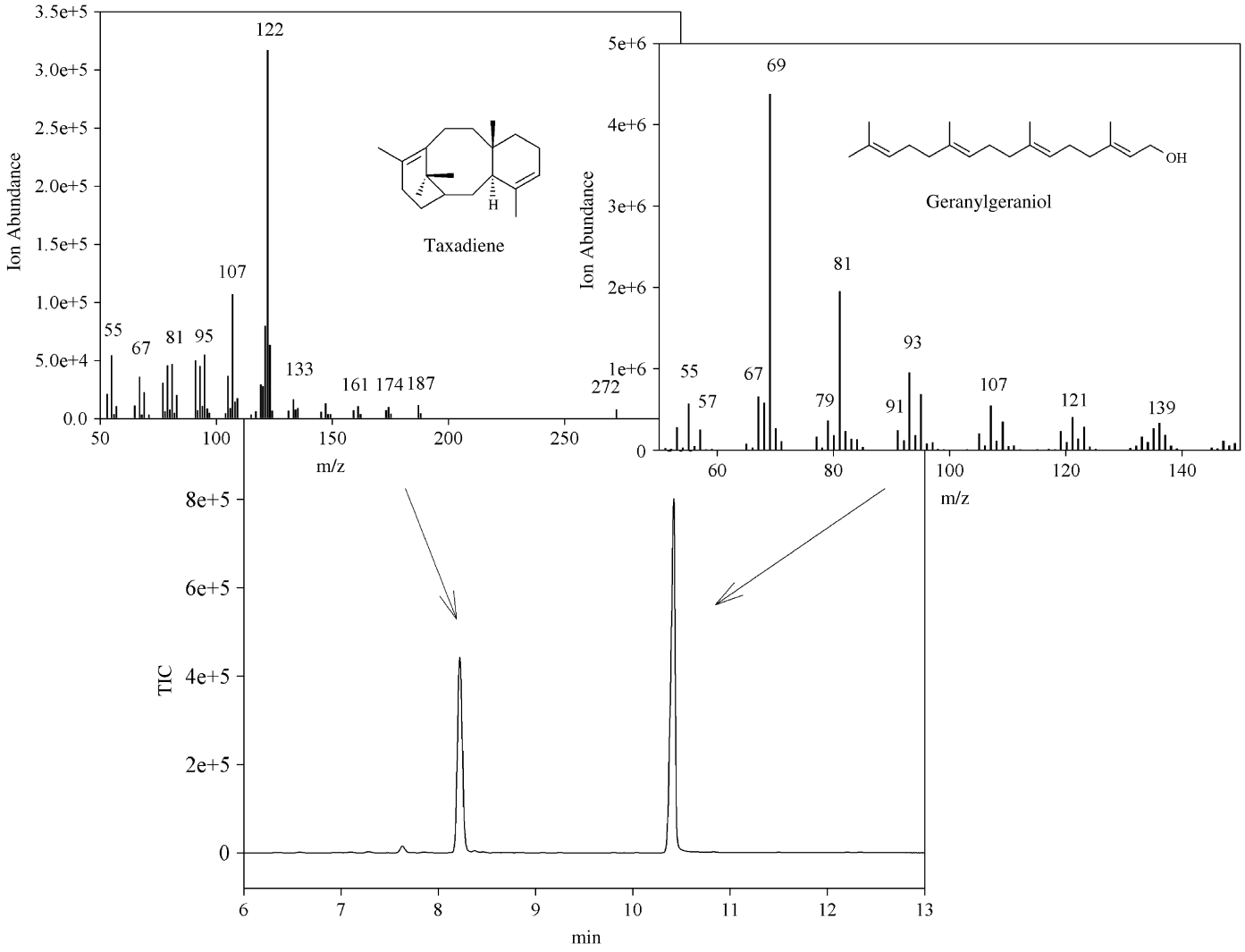


**Fig. 4.** GC–MS analysis of pentane extracts from recombinant *S. cerevisiae* strain CEN10 showing part of the total ion chromatogram, together with the mass spectra of taxa-4(5),11(12)-diene (retention time 8.2 min) and geranylgeraniol (retention time 10.5 min).

CEN10 showed a near 40-fold increase in taxadiene levels ($8.7 \pm 0.85$ mg/l) as well as significant amounts of geranylgeraniol ($33.1 \pm 5.6$ mg/l), showing potential for further increases in taxadiene production. For the first time, we have established a system for the efficient synthesis of taxadiene in yeast by establishing a sufficient supply of geranylgeranyl diphosphate, the common isoprenoid building block for all diterpenoids. Taxadiene is the first pathway committed intermediate in Taxol biosynthesis, providing a basis for the development of Taxol production in *S. cerevisiae*. The enhanced production of geranylgeraniol demonstrates the further potential for increasing flux into the taxadiene pathway in this system as shown in Fig. 4.

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## References

Amberg, C.D., Burke, D.J., Strathern, J.N., 2005. Methods in Yeast Genetics. Cold Spring Harbor Press, Cold Spring Harbor, NY, USA.

Andreason, A.A., Stier, T.J.B., 1953. Anaerobic nutrition of *Saccharomyces cerevisiae* I. Ergosterol requirements for growth in a defined medium. J. Cell. Comp. Physiol. 41, 23–26.

Baloglu, E., Kingston, D.G., 1999. The taxane diterpenoids. J. Nat. Prod. 62, 1448–1472.

Chang, M.Y., Keasling, J.D., 2006. Production of isoprenoid pharmaceuticals by engineered microbes. Nat. Chem. Biol. 3, 274–277.

Conolly, J.D., Hill, R.A., 1991. Dictionary of Terpenoids. Chapman & Hall, London.

Crowley, J.H., Leak Jr., F.W., Shianna, K.V., Tove, S., Parks, L.W., 1998. A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*. J. Bacteriol. 180, 4177–4183.

Davies, B.S.J., Wang, H.S., Rine, J., 2005. Dual activators of the sterol biosynthetic pathway of *Saccharomyces cerevisiae*: similar activation/regulatory domains but different response mechanisms. Mol. Cell. Biol. 25, 7375–7385.

DeJong, J.M., Liu, Y., Bollon, A.P., Long, R.M., Jennewein, S., Williams, D., Croteau, R.B., 2005. Genetic engineering of Taxol biosynthetic genes in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 93, 212–224.

Dimster-Denk, D., Thorsness, M.K., Rhine, J., 1994. Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Saccharomyces cerevisiae*. Mol. Biol. Cell. 5, 655–665.

Donald, K.A.G., Hampton, R.Y., Fritz, I.B., 1997. Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 63, 3341–3344.

Hefner, J., Rubenstein, S.M., Ketchum, R.E., Gibson, D.M., Williams, R.M., Croteau, R., 1996. Cytochrome P450-catalyzed hydroxylation of taxa-4(5),11(12)-diene to

taxa-4(20),11(12)-dien-5α-ol: the first oxygenation step in Taxol biosynthesis. Chem. Biol. 6, 479–489.
Hefner, J., Ketchum, R.E., Croteau, R., 1998. Cloning and functional expression of a cDNA encoding geranylgeranyl diphosphate synthase from *Taxus canadensis* and assessment of the role of this prenyltransferase in cells induced for Taxol production. Arch. Biochem. Biophys. 360, 62–74.
Kingston, D.G.I., 2007. The shape of the things to come: structural and synthetic studies of Taxol and related compounds. Phytochemistry 68, 1844–1854.
Koepp, A.E., Hezari, M., Zajicek, J., Vogel, B.S., LaFever, R.E., Lewis, N.G., Croteau, R., 1995. Cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene is the committed step of Taxol biosynthesis in pacific yew. J. Biol. Chem. 270, 8686–8690.
Lewis, T.L., Keesler, G.A., Fenner, G.P., Parks, L.W., 1988. Pleiotrophic mutations in *Saccharomyces cerevisiae* affecting sterol uptake and metabolism. Yeast 4, 93–106.
Lorenz, R.T., Parks, L.W., 1991. Involvement of heme components in sterol metabolism of *Saccharomyces cerevisiae*. Lipids 26, 598–603.
Jennewein, S., Long, R.M., Williams, R.M., Croteau, R., 2004a. Cytochrome P450 taxadiene-5α-hydroxylase, a mechanistically unusual monooxygenase catalyzing the first oxygenation step of Taxol biosynthesis. Chem. Biol. 11, 375–387.
Jennewein, S., Wildung, M.R., Chau, M., Walker, K., Croteau, R., 2004b. Random sequencing of an induced *Taxus* cell cDNA library for identification of clones involved in Taxol biosynthesis. Proc. Natl. Acad. Sci. USA 101, 9149–9154.
Lin, X., Hezari, M., Koepp, A.E., Floss, H.G., Croteau, R., 1996. Mechanism of taxadiene synthase, a diterpene cyclase that catalyzes the first step of Taxol biosynthesis in pacific yew. Biochemistry 35, 2968–2977.
Ohnuma, S., Suzuki, M., Nishino, T., 1994. Archaebacterial ether-linked lipid biosynthetic gene. Expression cloning, sequencing and characterization of geranylgeranyl diphosphate synthase. J. Biol. Chem. 269, 14792–14797.
Ro, D.K., Paradise, E.M., Ouellet, M., Fischer, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C., Withers, S.T., Shiba, Y., Sarpong, R., Keasling, J.D., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940–943.
Rosenberg, S., Coit, D., Tekamp-Olson, P., 1990. Glyceraldehyde-3-phosphate dehydrogenase-derived expression cassettes for constitutive synthesis of heterologous proteins. Methods Enzymol. 185, 341–351.
Schoendorf, A., Rithner, C.D., Williams, R.M., Croteau, R., 2001. Molecular cloning of a cytochrome P450 taxane 10β-hydroxylase cDNA from Taxus and functional expression in yeast. Proc. Natl. Acad. Sci. USA 98, 1501–1506.
Sikorski, P.S., Hiether, S., 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisisae*. Genetics 122, 19–27.
Stierle, A., Strobel, G., Stierle, D., 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of pacific yew. Science 260, 214–216.
Suffness, M., Wall, M.E., 1995. Discovery and development of Taxol. In: Suffness, M. (Ed.), Taxol Science and Applications. CRC Press, Boca Raton, FL, pp. 3–25.
van Dijken, J.P., Bauer, J., Brambilla, L., Duboc, P., Francois, J.M., Gancedo, C., Giuseppin, M.L., Heijnen, J.J., Hoare, M., Lange, H.C., Madden, E.A., Niederberger, P., Nielsen, J., Parrou., J.L., Petit, T., Porro, D., Reuss, M., van Riel, N., Rizzi, M., Steensma, H.Y., Verrips, C.T., Vindelov, J., Pronk, J.T., 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb. Technol. 26, 706–714.
Van Mullen, V., Wery, M., De Bolle, X., Vandenhaute, J., 2003. Construction of a set of *Saccharomyces cerevisiae* vectors designed for recombinant cloning. Yeast 20, 739–740.
Vik, Å., Rine, J., 2001. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 21, 6395–6405.
Walker, K., Croteau, R., 2001. Taxol biosynthetic genes. Phytochemistry 58, 1–7.
Wheeler, A.L., Long, R.M., Ketchum, R.E., Rithner, C.D., Williams, R.M., Croteau, R., 2001. Taxol biosynthesis: differential transformation of taxadien-5α-ol and its acetate ester by cytochrome P450 hydroxylases from *Taxus* suspension cells. Arch. Biochem. Biophys. 390, 265–278.
Williams, D.C., Wildung, R.M., Jin, A.O., Dalal, D., Oliver, J.S., Coates, R.M., Croteau, R., 2000. Heterologous expression and characterization of a "pseudomature" form of taxadiene synthase involved in paclitaxel (Taxol) biosynthesis and evaluation of a potential intermediate and inhibitor of the multistep diterpene cyclization reaction. Arch. Biochem. Biophys. 379, 137–146.
Wildung, M.R., Croteau, R., 1996. A cDNA clone for taxadiene synthase, the diterpene cyclase that catalyzes the committed step of Taxol biosynthesis. J. Biol. Chem. 271, 9201–9204.