Synthetic Biology

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Brewing Painkillers: A Yeast Cell Factory for the Production of Opioids from Sugar

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For hundreds of years, humans have utilized the yeast *Sacharomyces cerevisiae* to leaven bread and brew alcoholic beverages. Meanwhile, *S. cerevisiae* has become an important eukaryotic cell factory that is utilized in many biotechnological applications for producing enzymes and chemicals. Smolke and co-workers succeeded in creating the first yeast cell factory for transforming D-glucose into the opioid painkillers thebaine and hydrocodone.[1]

The author's approach to achieve this goal was to apply synthetic biology (SynBio). Whereas current biocatalysis employs classical recombinant DNA technology and focuses on the utilization of single or few enzymes for distinct biotransformations, SynBio aims at constructing rationally designed complex systems, such as organisms featuring completely novel metabolic pathways.[2] Opioid alkaloids are complex functionalized tertiary amines, and their synthesis through the SynBio approach highlights the potential of this technology. Over a decade of work resulted in a yeast strain that artificially expresses 23 genes originating from yeast, plants such as the opiate producer *Papaver somniferum,* bacteria, and rat. This work currently constitutes the largest synthetically assembled metabolic pathway published. The elegance of this system—compared to numerous attempts of chemical total syntheses of opioids[3]—lies in the fact that it can be seen as a one-pot reaction. No intermediate work-up and purification steps are necessary, and glucose and simple amino acids are used as the sole starting materials and reagents to build all of the necessary catalysts. In this Highlight, we summarize the main achievements and challenges of this study, which demonstrates the high potential but also the hurdles for the current SynBio technology.

Building the opioid skeleton requires the combination of several amine-manipulating enzymes (Scheme 1). The key steps involve 1) an asymmetric Pictet–Spengler reaction to form the tetrahydroisoquinoline skeleton of Norcoclaurine (module III), 2) after subsequent hydroxylation and methylation steps, the tertiary amine (*S*)-reticuline undergoes a stereo inversion (module VI), and 3) the tetracyclic salutaridine is formed by intramolecular C–C-coupling (module VI). The subsequent reduction and tautomerization steps furnish the opioid hydrocone.

The researchers took a modular approach characteristic for SynBio: *S. cerevisea* was supplied with seven DNA modules encoding 2–7 enzymes each (Scheme 1). The design of the modules reflects how Smolke's group responded to the challenges of constructing a complex pathway:

1) Metabolites originating from primary metabolism have to be provided in sufficient amounts to direct the metabolic flux into the target products. Module I ensures conversion of glucose into L-tyrosine and 4-hydroxyphenylacetaldehyde (4-HPAA) as important precursors. In future applications, such a yeast strain could be used as a basic framework to which additional "plug-in" modules can be added for the production of other compounds built from tyrosine.
2) Four of the seven modules enable a series of transformations leading to metabolite structures that represent a branching point in the biosynthesis. This once again underlines the modular concept, with use of functional entities that can be re-utilized. In this example, strains producing norcoclaurine, reticuline, and thebaine could be expanded by additional modules to synthesize other derivatives of the isoquinoline alkaloid family.
3) Just one enzymatic step in the whole pathway required tetrahydrobiopterin (BH4) as a cofactor, which is not available in *S. cereviseae*. A BH4-biosynthesis module was thus designed, which could later be used for engineering other BH4-dependent pathways in a "plug and play" fashion.
4) To ensure sufficient flux through all of the steps of the synthetic pathway, bottlenecks have to be overcome. Module V supplied higher levels of three rate-limiting enzymes to reduce the accumulation of three intermediates, which were identified by flux analysis.

Another major achievement in this project was the identification of the amino acid sequences of reticuline isomerase (independently recently published by Farrow et al.),[4] which represents the long-sought missing link to connect previously developed steps towards opioid synthesis in yeast. Stereoinversion is achieved by a two-step reaction:

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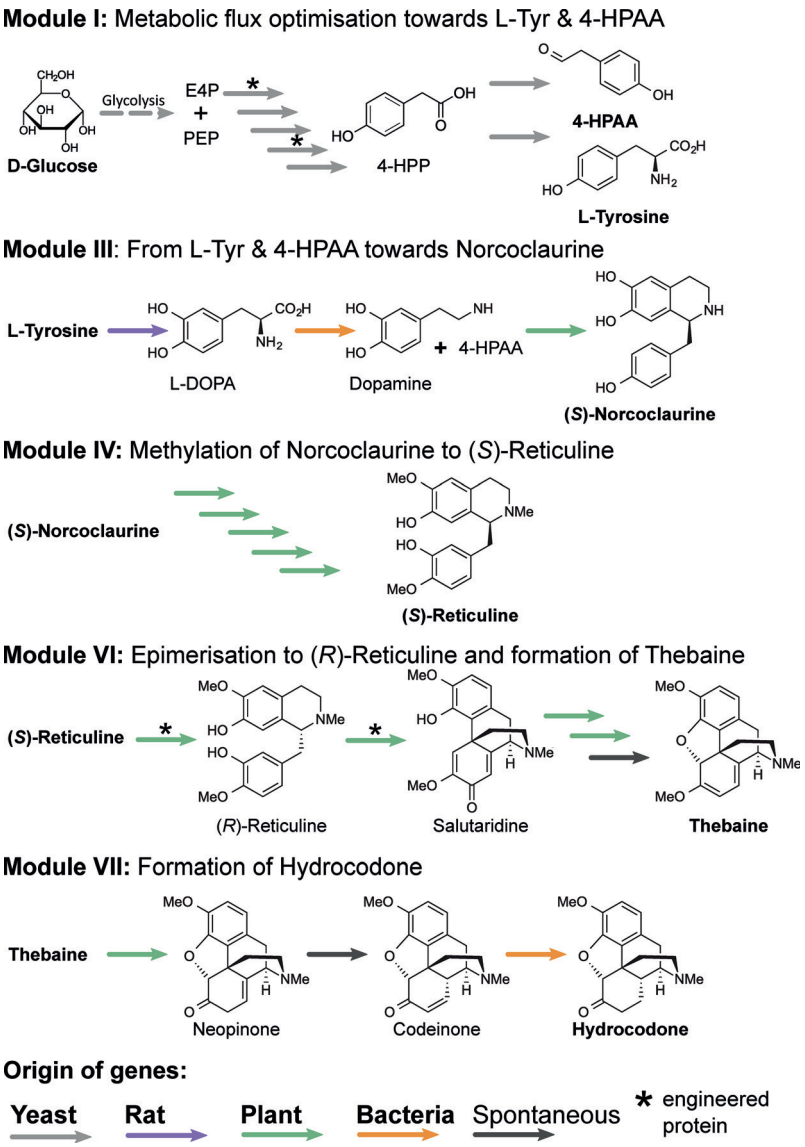
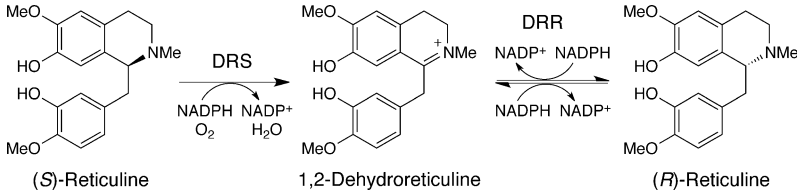


Scheme 1. The SynBio modules constructed for the synthesis of opioids from glucose. E4P = erythrose-4-phosphate, PEP = phosphoenolpyruvate.

(*S*)-reticuline reacts in a P450 oxidase-catalyzed step with molecular oxygen to form an imine intermediate. An NADP-dependent reductase subsequently forms the *R* isomer (Scheme 2). The discovered isomerase is a natural fusion protein and contains both activities. In contrast to racemization, this process is unidirectional, since both steps are highly stereospecific and allow only conversion of the *S* enantiomer into the *R* enantiomer.[4] Interestingly, a similar cascade for deracemization of secondary amines was recently published by Turner's group: an engineered monoamine oxidase (MAO) was coupled with an imine reductase (IRED),[5] thereby giving access to both amine enantiomers by applying stereocomplementary enzymes. IREDs and MAOs are currently under investigation as important tools for the biocatalytic preparation of chiral amines.[6]



Scheme 2. Two-step stereoinversion of reticuline by a fusion protein containing two catalytic domains. DRS = 1,2-dehydroreticuline synthase domain, DRR = 1,2-dehydroreticuline reductase domain.

A second key success was the engineering of the rate-limiting enzyme salutaridine synthase (SalSyn). Extensive analysis revealed an unfavorable orientation of SalSyn in the endoplasmatic reticulum (ER), which results in reduced availability for biocatalysis. The issue was addressed by creating a rationally designed library of chimeric proteins consisting of N termini of varying length from plant and bacterial P450 monooxygenases that were previously shown to be correctly inserted into the ER membrane of yeast, and fusing them to the catalytic domain of the SalSyn.

The largest still-open challenge in this proof-of-concept study is optimization of the production titers: Starting from 20 $g L^{-1}$ glucose, 6.4 $\mu g L^{-1}$ thebaine and 0.3 $\mu g L^{-1}$ hydrocodone could be produced, respectively. These yields are orders of magnitude away from the 5 $g L^{-1}$ threshold that is considered to be the minimum titer to give a feasible alternative to poppy farming. We missed a thorough analysis of the reasons for this low titer and a discussion of how this could be improved. Other SynBio approaches, such as yeast-based production of the antimalaria-drug precursor artemisininic acid, have successfully tackled similar challenges.[7] In this case, the company Amyris applied a combination of systems biology (computational prediction of fluxes), metabolic engineering, and bio- and chemical-process development to increase yields from 100 mg L^{-1} to 25 g L^{-1} within few years.[8]

Replacing traditional plant-based production processes with SynBio-based microbial processes offers the advantages of shorter production times (days vs. a year for the annual poppy plant), independence from external factors such as climate, and high consistency across batches. According to The World Health Organization[1] developing countries in particular have limited to no access to required painkillers, and an alternative source of painkillers could ease this problem. This study by Galanie et al.[1] represents an important step in this direction, showing that scientists can develop solutions to tackle global problems. The challenge remains of how we can ensure that the results from these efforts will benefit society, in this case especially people living in developing countries that urgently need painkillers. Furthermore, when replacing a traditional plant-based production process with a SynBio-based microbial process, an impact on the markets is inevitable. A story with many parallels is that of yeasts that produce artimisinic acid. While this is an important step towards fighting malaria, it threatens to put many sweet wormwood (*Artesemia annum*) farmers in developing, malaria-ridden nations out of business.[9]

As with all disruptive technologies, synthetic biology needs to be evaluated in respect to its impact on society, even more so if it enables the production of substances prone to illicit use. The publication touches on this point by describing the stipulations for a laboratory working with narcotic-producing organisms. This includes background screening of the involved scientists, increased containment of yeast strains, and laboratory security, as well as tight control through the U.S. Drug Enforcement Agency.

Synthetic biology is the next step in the evolution of genetic engineering, and it will enable us to produce advanced biofuels, basic chemicals, pharmaceuticals, and fine chemicals in a sustainable fashion. Dr. Smolke and her team are pursuing commercialization of their process through their all-female-led company Antheia (the greek goddess of flowers).

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