Combinatorial Biosynthesis of Natural and Non-natural Plant-derived Phenols in Microorganisms

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„Gebt mir einen festen Punkt – und ich werde die Welt aus den Angeln heben.“

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Table of contents

Abstract

Phenylpropanoids and phenylpropanoid-derived plant polyphenols have numerous applications in pharmaceutical and food industries and are used for example as antibiotics, therapeutics and colourants. Unfortunately, their extraction from plants is not efficient due to low product concentrations. In addition, downstream processing is impeded because the desired phenylpropanoids are present in a complex mixture of compounds with very similar chemical properties. These limitations can be overcome using microbial platform organisms to produce phenylpropanoids and polyphenols. The following results were obtained in this thesis:

(1) The efficient production of monolignols is an important requirement for the synthesis of the pharmacologically relevant lignans, which belong to the class of plant polyphenols. An engineered *Escherichia coli* strain, equipped with a set of genes encoding enzymes for monolignol synthesis, was used to elucidate the microbial production of different monolignols. For this purpose, natural and non-natural phenylpropanoids were supplemented to respective biotransformations using the engineered strain to examine the promiscuity of heterologous enzymes. It was revealed that the engineered strain was able to catalyse the stepwise reduction of six naturally occurring phenylpropanoids including 5-hydroxyferulic acid and sinapic acid, which was reported for the first time. Additionally, chemically interesting non-natural phenylpropanoids, such as 3,4,5-trimethoxycinnamic acid, 5-bromoferulic acid, 3-nitroferulic acid, and a bicyclic *p*-coumaric acid derivative were also reduced to the corresponding non-natural monolignols. The microbial production of these compounds is a good basis for the synthesis of more complex plant-derived compounds.

(2) The site-specific and stereospecific decoration of phenylpropanoids with hydroxyl and *O*-methyl groups is a good possibility to obtain a variety of different phenylpropanoids. First a 4-coumarate 3-hydroxylase (C3H) from *Saccharothrix espanaensis* was used for the hydroxylation of *p*-coumaric acid yielding caffeic acid. In a second step, this enzyme was combined with the previously used monolignol pathway to produce caffeoyl alcohol from supplemented *p*-coumaric acid. Biotransformations resulted not only in caffeoyl alcohol production but also in *p*-coumaryl alcohol production. This underlined the challenges of selective product synthesis with promiscuous enzymes. Further substitutions at the phenyl ring were introduced with the caffeic acid *O*-methyltransferase (COMT) from *Medicago sativa*, which was engineered towards the methylation of caffeic acid and 5-hydroxyferulic acid in whole cell biotransformations with engineered *E. coli* strains. COMT libraries obtained from site-saturation mutagenesis at four positions were screened and selected strains were further characterised. Amino acid substitutions in four positions located in or adjacent to the methoxy binding pocket were shown to alter the substrate specificity towards caffeic acid and 5-hydroxyferulic acid to produce ferulic acid and sinapic acid, respectively. Variants were found with an increased productivity of up to 17 % for ferulic acid production and 46 % for sinapic acid production compared to the not engineered wild type *E. coli* strain. The results contribute to a better understanding of these enzymes and will help to perform metabolic engineering for the microbial production of more complex phenylpropanoid-derived compounds in the future.

(3) The multicopper oxidase CueO from *Rhodococcus erythropolis*, previously used for the *in vitro* production of lignan, was now examined regarding lignan production using a respective recombinant *E. coli* strain. The gene *cueO* was expressed under the control of a T7 expression system and a pBAD expression system to find the best suited induction conditions. It was revealed that the tight gene regulation of the pBAD expression system can be used for the microbial lignan production. The strain was cultivated in the presence of coniferyl alcohol (1 mM) and produced 0.17 mM (+)-pinoresinol. This is the first time, that microbial (+)-pinoresinol production was observed.

Zusammenfassung

Phenylpropanoide und von ihnen abgeleitete pflanzliche Polyphenole haben zahlreiche Anwendungen in der Pharma- und Nahrungsmittelindustrie und werden beispielsweise als Antibiotika, Therapeutika und Farbstoffe eingesetzt. Leider ist ihre Extraktion aus Pflanzen ineffizient, da sie nur in geringen Konzentrationen vorkommen. Außerdem wird die Produktaufarbeitung dadurch erschwert, dass die gewünschten Phenylpropanoide im Pflanzengewebe in einem komplexen Stoffgemisch mit ähnlichen chemischen Eigenschaften vorkommen. Diese Einschränkungen können durch die Nutzung von mikrobiellen Plattformorganismen überwunden werden. Im Rahmen dieser Arbeit wurden folgende Ergebnisse erzielt:

(1) Die effiziente Monolignolproduktion ist eine wichtige Voraussetzung für die Synthese des pharmakologisch interessanten Lignans, welches ein pflanzliches Polyphenol ist. Ein genetisch veränderter *Escherichia coli* Stamm wurde mit Enzymen für die Monolignolproduktion ausgestattet, um die Möglichkeit der Produktion einer Vielzahl von unterschiedlichen Monolignolen zu untersuchen. Dafür wurden natürliche und nicht-natürliche Phenylpropanoide zu Biotransformationen hinzugegeben und die Promiskuität der Enzyme erforscht. Es konnte gezeigt werden, dass der genutzte Stamm die schrittweise Reduktion von sechs natürlichen Phenylpropanoide katalysiert, darunter auch die Reduktion von 5-Hydroxyferulasäure und Sinapinsäure, welches zum ersten Mal beschrieben werden konnte. Die chemisch interessanten nicht-natürlichen Phenylpropanoide 3,4,5-Trimethoxyzimtsäure, 5-Bromferulasäure, 3-Nitroferulasäure und ein bizyklisches *p*-Coumarsäurederivat wurden ebenfalls zum entsprechenden Monolignol reduziert. Die mikrobielle Produktion dieser Stoffe ist eine gute Basis für die Synthese von komplexeren pflanzlichen Substanzen.

(2) Die Dekoration von Phenylpropanoiden mit Hydroxy- und *O*-Methylgruppen ist eine gute Möglichkeit, um eine Vielzahl verschiedener Phenylpropanoide zu erhalten. Zunächst wurde eine 4-Cumarat-3-Hydroxylase (C3H) von *Saccharothrix espanaensis* für die Hydroxylierung von *p*-Cumarsäure zu Kaffeesäure verwendet. In einem zweiten Schritt wurde dieses Enzym mit dem zuvor verwendeten Monolignolsyntheseweg kombiniert, um Kaffeoylalkohol aus hinzugefügter *p*-Cumarsäure herzustellen. Biotransformationen führten nicht nur zur Produktion von Kaffeoylalkohol, sondern auch zur Produktion von *p*-Cumarylalkohol. Dies unterstreicht die Herausforderungen der selektiven Produktsynthese mit promiskuitiven Enzymen. Weitere Substitutionen am Phenylring wurden mit einer Kaffeesäure *O*-Methyltransferase (COMT) aus *Medicago sativa* eingefügt. Diese wurde gentechnisch verändert, um die Methylierung von Kaffeesäure und 5-Hydroxyferulasäure in Ganzzellbiotransformationen mit *E. coli* zu steigern, wodurch Ferulasäure bzw. Sinapinsäure entsteht. Durch ortsspezifische Sättigungsmutagenese an vier Positionen wurde eine COMT Bibliothek erstellt, anschließend durchmustert und ausgewählte Varianten charakterisiert. Es konnte gezeigt werden, dass Aminosäuresubstitutionen in den Position 135, 136, 162 und 172, die in der Methoxybindetasche oder in deren Nähe lokalisiert sind, die Substratspezifität verändert. Außerdem konnte die Produktivität im Vergleich zur Wildtypvariante um 17 % für die Ferulasäureproduktion bzw. um 46 % für die Sinapinsäureproduktion gesteigert werden. Die erhaltenen Varianten können zu einem besseren Verständnis dieser Enzyme beitragen und eine gentechnische Veränderung von Organismen für die Produktion von komplexen Phenylpropanoid-Verbindungen erleichtern.

(3) Die Multikupferoxidase CueO aus *Rhodococcus erythropolis* wurde bisher für die *in vitro* Lignanproduktion genutzt und wurde nun hinsichtlich der Verwendung in *E. coli* untersucht. Das Gen *cueO* wurde unter der Kontrolle des T7-Expressionssystems und des pBAD-Expressionssystems exprimiert, um die am besten geeigneten Induktionsbedingungen zu finden. Es zeigt sich, dass die enge Genregulation durch das pBAD-Expressionssystem für die mikrobielle Lignanproduktion verwendet werden kann. Der konstruierte Produktionsstamm wurde in Gegenwart von 1 mM Coniferylalkohol kultiviert und die Konzentration des Lignans (+)-Pinoresinol wurde bestimmt. Am Ende der Biotransformation wurden unter Verwendung dieses Enzyms *in vivo* 0.17 mM (+)-Pinoresinol hergestellt. Dies ist das erste Mal, dass die Produktion von mikrobiellem (+)-Pinoresinol beobachtet wurde.

Abbreviations

4CL	4-coumarate: CoA ligase
A	adenine
ara	arabinose
ATP	adenosine triphosphate
C	cysteine
C3H	*p*-coumarate 3-hydroxylase
C4H	cinnamate 4-hydroxylase
CAD	cinnamyl alcohol dehydrogenase
CCR	cinnamyl-CoA reductase
CHI	chalcone isomerase
CHS	chalcone synthase
CoA	coenzyme A
COMT	caffeic acid *O*-methyltransferase
CueO	cuprous oxidase
Da	Dalton
DIR	dirigent protein
E	glutamic acid
F	phenylalanine
F5H	ferulate 5-hydroxylase
G	glutamine
HPLC	high performance liquid chromatography
I	isoleucine
IPTG	isopropyl-β-D-thiogalactopyranoside
L	leucine
LAC	laccase
LB	lysogeny broth
LC/MS	liquid chromatography/mass spectrometry
leu	leucine
K	lysine
Kan	kanamycin
M	molar (mol/L)
MON	cytochrome P450 monooxygenase

NF-κB	nuclear factor-kappa B
OD	optical density
OMT	*O*-methyltransferase
p	*para*
PAL	phenylalanine ammonia lyase
Q	glutamine
R	arginine
S	serine
SAH	*S*-adenosyl-L-homocysteine
SAM	*S*-adenosyl-L-methionine
SDS	sodium dodecyl sulphate
SIM	selective Ion Monitoring
STS	stilbene synthase
TAL	tyrosine ammonia lyase
T	threonine
V	valine

1 Scientific context and key results of this thesis

1.1 Polyphenols - naturally occurring and application

Natural polyphenols comprise a multitude of plant-derived secondary metabolites which can serve as therapeutics, antibiotics and colourants (Licciardi and Underwood, 2011; Marienhagen and Bott, 2013; Mi et al., 2016). Some polyphenols have antioxidant properties and reduce the risk of cancer, others are added to processed food products to make up for colour losses during processing (Guo et al., 2009; Sowbhagya and Chitra, 2010; Tan et al., 2011). In plants polyphenols are synthesised by enzymes of the phenylpropanoid pathway from L-phenylalanine and L-tyrosine as key building blocks. The such produced product range includes various phenylpropanoids, like cinnamic acid derivatives (other phenylpropanoids in Fig. 1) and monolignols (*p*-coumaryl alcohol in Fig. 1), which are precursor molecules for polyphenols.

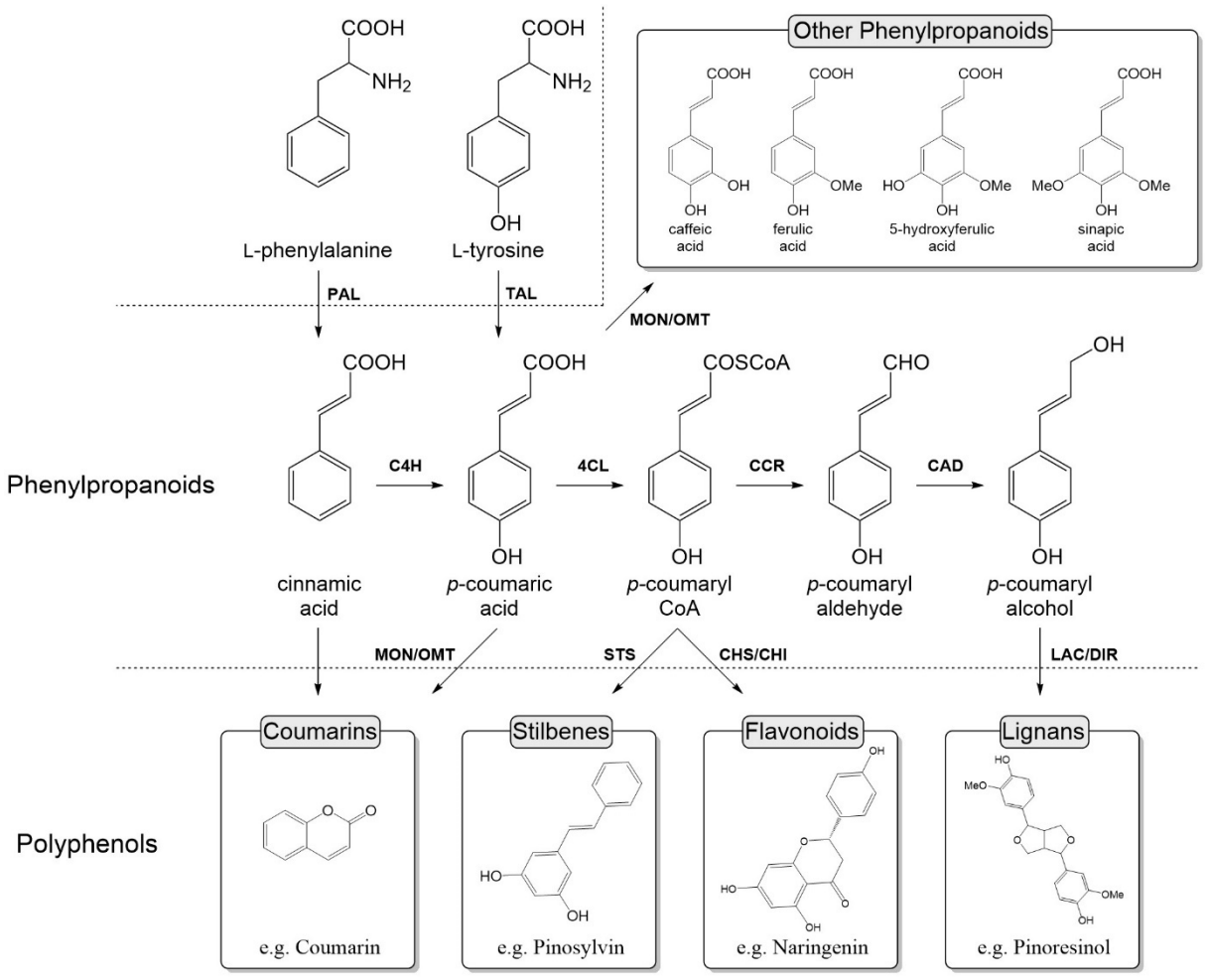

Fig. 1 Overview of biosynthetic pathways towards various phenylpropanoid-derived polyphenols. The amino acids L-tyrosine and L-phenylalanine serve as precursors for the

phenylpropanoids cinnamic acid and *p*-coumaric acid, which are formed by non-oxidative deamination. These compounds are building blocks of coumarins. Hydroxylation and *O*-methylation in *meta*-position of the phenyl ring leads to other phenylpropanoids like caffeic acid or ferulic acid. The propene tail of *p*-coumaric acid, as part of the phenylpropanoid core structure, is modified in the first step by a 4-coumarate: CoA ligase. The resulting phenylpropanoyl-CoA thioester is a building block of stilbenes and flavonoids. Furthermore, the thioester can be reduced in two steps to *p*-coumaryl alcohol, a monolignol and lignan precursor. 4CL: 4-coumarate: CoA ligase, C4H: cinnamate 4-hydroxylase, CAD: cinnamyl alcohol dehydrogenase, CCR: cinnamyl-CoA reductase, CHI: chalcone isomerase, CHS: chalcone synthase, DIR: dirigent protein, LAC: laccase, MON: cytochrome P450 monooxygenase, OMT: *O*-methyltransferase, PAL: phenylalanine ammonia lyase, STS: stilbene synthase, TAL: tyrosine ammonia lyase.

Coumarins are secondary metabolites from plants, derived from cinnamic acid or *p*-coumaric acid and contain a 1,2-benzopryone backbone (Fig. 1). They natural function as iron chelators in the soil or as defensive compounds against pathogens (Yang et al., 2015). Moreover, they can be found in tonka beans and are used as flavouring agents in alcoholic beverages and food (Wang et al., 2013).

p-Coumaryl CoA serves as precursor for two polyphenol classes: stilbenes and flavonoids. One representative for stilbenes is pinosylvin (Fig. 1), which is present in the heartwood of *Pinaceae* and makes it resistant towards fungal attack (Hovelstad et al., 2006). Recent studies revealed pinosylvin as beneficial for human health with anti-inflammatory and anti-cancer properties (Laavola et al., 2015; Liang et al., 2016). Another stilbene is resveratrol (Fig. 1) which can be found amongst others in grapes, berries, and peanuts (Shin et al., 2012). It is produced in response to injury or during an attack by bacteria or fungi (Frémont, 2000). Furthermore, resveratrol can presumably slow down the progression or even prevent cardiovascular diseases, cancer and showed positive impact on the lifespans of various organisms (Schmidlin et al., 2008; Shukla and Singh, 2011).

Naringenin (Fig. 1) is a representative of the class of flavonoids and can be found in a variety of fruits, especially grapefruit and herbs (Felgines et al., 2000; Yáñez et al., 2007). Among other positive characteristics, it stimulates DNA repair in prostate cancer cells and may prevent mutagenic changes (Gao et al., 2006). In a different study, naringenin was orally administered to mice once per day for 31 days to test its potential to treat Alzheimer's Disease. It could be shown that the memory was improved and the formation of harmful amyloid and tau proteins was reduced (Yang et al., 2017).

Lignans, also correlated with numerous health benefits, result from the oxidative coupling of two monolignols (e.g. *p*-coumaryl alcohol in Fig. 1, coniferyl alcohol in Fig. 5) by laccases and dirigent proteins. As an example, pinoresinol, a dilignol consisting of two coniferyl alcohol molecules, is known for its broad medical application (Adlercreutz, 2007; Cornwell et al., 2004; Dixon, 2004; Duncan, Alison et al., 2003). It has antifungal properties (Hwang et al., 2010; Kim et al., 2010; Wikul et al., 2012) and reduces the risk of cardiovascular diseases and hormone-dependent cancer (Adlercreutz, 2007; Adolphe et al., 2010; Ayella et al., 2010; Azrad et al., 2013; Peterson et al., 2010). Moreover it has cytotoxic effects on tumour cells and an inhibitory effect on HIV-1 replication (López-Biedma et al., 2016; Mitsuhashi et al., 2008; Moon et al., 2008). A more complex derivative of pinoresinol is podophyllotoxin, which is derived by a series of oxidation-, reduction- and methylation steps (Canel et al., 2000). It can be used for the treatment of venereal wart and its semi synthetic derivatives are used in cancer therapy (Davin and Lewis, 2005).

1.2 Phenylpropanoids - naturally occurring and application

Cinnamic acid is the “simplest” phenylpropanoid and consists of a phenyl ring attached to a propene tail (Buono et al., 2018) (Fig. 1). It is obtained from cinnamon oil and is mainly used as an antioxidant and preserving agent in food industry (Li et al., 2014; Sun et al., 2018; Zanetti et al., 2015). Cinnamic acid can be reduced to the corresponding monolignol cinnamyl alcohol, a building block for lignan synthesis in plants, and this compound is present only in small amounts in natural sources (Zucca et al., 2009). Therefore, chemical synthesis is used to produce sufficient amounts needed for fragrance and flavour ingredients (Bickers et al., 2005).

p-Coumaric acid, a cinnamic acid derivative, harbours an additional hydroxyl group in 4’-position of the phenyl ring (Fig. 1). It can be found in numerous plants like tomatoes and carrots but also in wine and vinegar (Carrero Gálvez et al., 2014). Its application includes the prevention of UV-induced damage to eye tissue and artery diseases (Ilavenil et al., 2016). *p*-Coumaryl alcohol is the respective monolignol and plays a key role in lignin formation, which is important for water transport in plants and prevents the degradation of wall polysaccharides being a major line of defence against pathogens, insects, and other herbivores (Hatfield and Vermerris, 2001).

Another cinnamic acid derivative is caffeic acid with a dihydroxylated phenyl ring (Fig. 1). It is a natural ingredient in coffee beans and found in apples, pears, bell peppers,

and mate tea (Bojić et al., 2013). In *Aspergillus flavus* caffeic acid can impede oxidative stress, which would otherwise result in triggered or enhanced aflatoxin production. This is detrimental because even the production of very low hepato-carcinogenic aflatoxins quantities can have a huge negative impact on food safety and on the quality of several agricultural products (Kim et al., 2008). Caffeoyl alcohol can be found in several vanilla and cacti species and is of interest in plant science, food industry and bioenergy research (Chen et al., 2012; Liu et al., 2017).

Ferulic acid is ubiquitous in plants as the most abundant phenolic acid and it is one of the effective components in Chinese medicine herbs (Ou and Kwok, 2004). Due to its structural features it exhibits antioxidative activity. For instance, the hydroxyl group at the phenyl ring is responsible to capture and neutralize reactive oxygen species and the *O*-methyl group at the phenyl ring is important for the stability of the molecule forming a hydrogen bond with the hydroxyl group (Graf, 1992) (Fig. 1). It was demonstrated that ferulic acid has an effect against erythema, photoaging and skin cancer, which are caused by UV radiation. Moreover, the chemical stability of vitamins were increased by the incorporation of ferulic acid in topical solutions containing vitamins (Peres et al., 2018). The corresponding monolignol coniferyl alcohol is present in lignin from dicotyledonous angiosperms (Ruelland et al., 2003) and is a precursor molecule for pinoresinol, a putative hypoglycaemic agent in defatted sesame seeds (Wikul et al., 2012). Furthermore, coniferyl alcohol was also identified as a honey bee queen retinue pheromone (Keeling et al., 2003).

Another cinnamic acid derivative is 5-hydroxyferulic acid, carrying two hydroxyl groups and one *O*-methyl group at the phenyl ring (Fig. 1). It is commonly found in plants and responsible for plant growth. For example, seedlings which were grown on media containing 5-hydroxyferulic acid were more vigorous than seedlings grown on ferulic acid media (Chapple et al., 1992). 5-Hydroxyconiferyl alcohol is also involved in lignification and can be found in several angiosperm plants (Parvathi et al., 2001).

Sinapic acid is another cinnamic acid derivative and harbours one hydroxyl group and two *O*-methyl groups at the phenyl ring (Fig. 1). It is prevalent in plants including fruits (lemon, strawberries), vegetables (broccoli, cabbage), and herbs (borage, rosemary, thyme) (Nićiforović and Abramovič, 2014). Previous studies elucidated that sinapic acid inactivates the nuclear factor-kappa B (NF-κB), which suppresses the expression of pro-inflammatory mediators such as tumour necrosis factor-α and interleukin-1β

(Yun et al., 2008). NF-κB is important for the immune response to infections and its defective regulation is linked to autoimmune diseases, cancer, and improper immune development (Shukla and Singh, 2011). Sinapyl alcohol is the precursor for syringyl lignin units and is necessary for lignin formation and quality (Boerjan et al., 2003; Do et al., 2007).

1.3 Enzymes participating in the phenylpropanoid pathway and polyphenol synthesis

Enzymes involved in the phenylpropanoid metabolism have a broad substrate range and can convert a plurality of different substrates. Their activities target modifications of the propene tail, the phenyl group or contribute to polyphenol assembly (Marienhagen and Bott, 2013).

1.3.1 Modulations at the propene tail

Phenylpropanoids are precursor molecules of polyphenols and are derived from the aromatic amino acids L-phenylalanine or L-tyrosine. These amino acids are non-oxidatively deaminated by ammonia lyases, for example a tyrosine ammonia lyases (TAL) is needed for the deamination of L-tyrosine (Fig. 2).

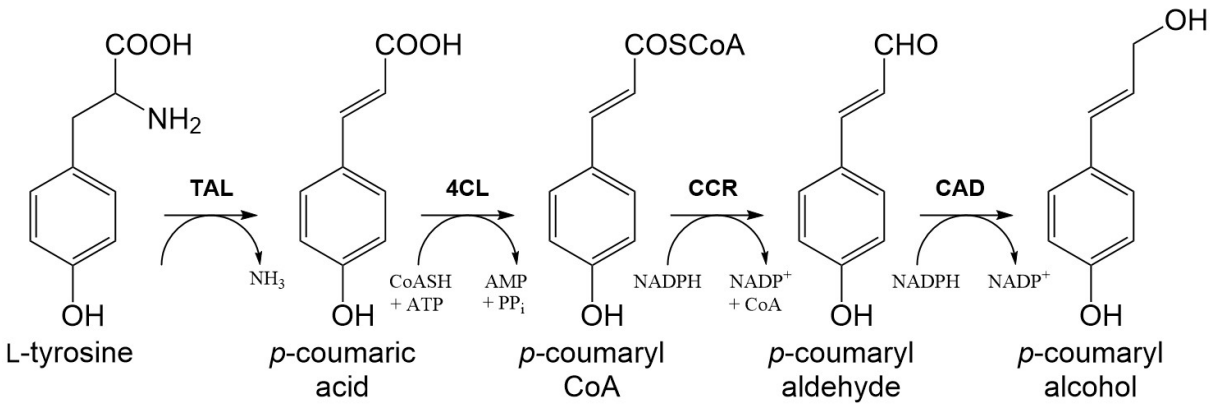

Fig. 2 Biosynthetic pathway for *p*-coumaryl alcohol synthesis from L-tyrosine. *p*-Coumaric acid is formed by deamination of L-tyrosine and is further modified by a 4-coumarate: CoA ligase to the corresponding phenylpropanoyl-CoA thioester. The thioester is reduced in two steps by a reductase and an alcohol dehydrogenase first to an aldehyde and then to *p*-coumaryl alcohol. TAL: tyrosine ammonia-lyase, 4CL: 4-coumaryl-CoA ligase, CCR: cinnamyl-CoA reductase, CAD: cinnamyl alcohol dehydrogenase.

The outcome is a phenyl group attached to a propene tail, which is known as the phenylpropanoid core structure. (Jansen et al., 2014; Marienhagen and Bott, 2013; Milke et al., 2018). In case of TAL, L-tyrosine is deaminated to *p*-coumaric acid, which

is coenzyme A (CoA)-activated by 4-coumarate-CoA ligases (4CL) in the next step. Afterwards, *p*-coumaryl CoA is reduced to *p*-coumaryl aldehyde by a cinnamoyl-CoA reductase (CCR). The last step in this pathway is catalysed by a cinnamyl alcohol dehydrogenase (CAD), which reduces *p*-coumaryl aldehyde to *p*-coumaryl alcohol, also known as monolignol. Monolignols are building blocks for the lignin synthesis in plants, but are also important for the synthesis of the pharmaceutically interesting group of lignans (Korkina et al., 2011; Neutelings, 2011). Enzymes participating in the monolignol pathway are known for their broad substrate spectrum and might be used for the production of other monolignols besides *p*-coumaryl alcohol (Ferrer et al., 2008).

1.3.2 Modification at the phenyl group

In addition to the pathway from *p*-coumaric acid to *p*-coumaryl alcohol, there is also the possibility to convert *p*-coumaric acid to sinapic acid. The biosynthesis of various cinnamic acid derivatives starts with the hydroxylation of cinnamic acid catalysed by a cinnamate 4-hydroxylases (C4H) (Fig. 3).

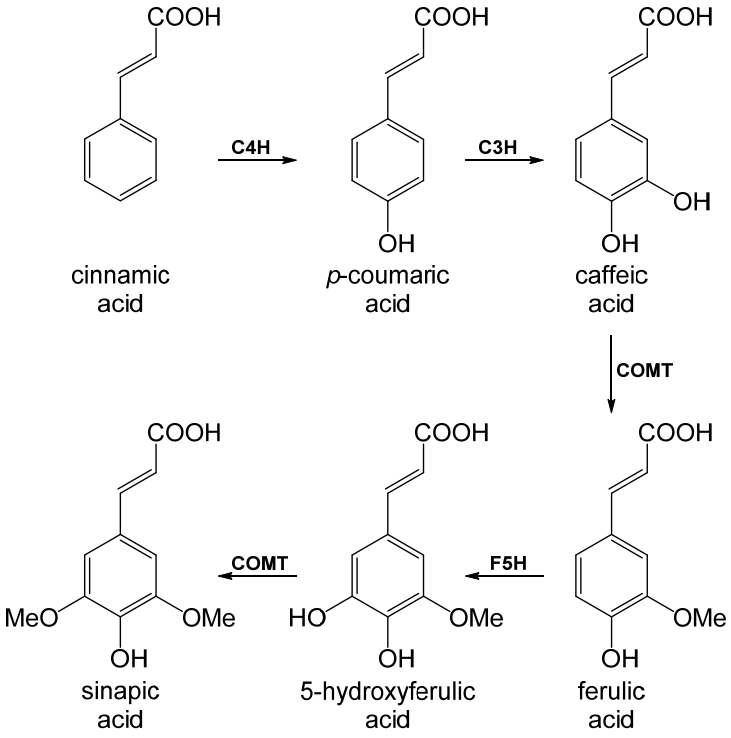

Fig. 3 Pathway of cinnamic acid derivatives. In the first step, cinnamic acid is hydroxylated to *p*-coumaric acid. Further phenylpropanoids are built by subsequent hydroxylation and *O*-methylation steps in *meta*-position of the phenyl ring. C4H: cinnamate 4-hydroxylase, C3H: 4-coumarate 3-hydroxylase, COMT: caffeic acid *O*-methyltransferase, F5H: ferulate 5-hydroxylase.

This is followed by an additional hydroxylation catalysed by a 4-coumarate 3-hydroxylase (C3H). The product of this reaction is caffeic acid, which is *O*-methylated at this hydroxyl group by a caffeic acid *O*-methyltransferase (COMT). The resulting ferulic acid is then hydroxylated in 5'-position by a ferulate 5-hydroxylase (F5H) yielding 5-hydroxyferulic acid. The subsequent methylation at this hydroxyl group leads to sinapic acid and is catalysed by COMT.

In this thesis, C3H and COMT were used for the microbial production of caffeic acid, ferulic acid, and sinapic acid in *E. coli*, and are therefore here described in detail.

4-Coumarate 3-hydroxylase (C3H)

The conversion of *p*-coumaric acid to caffeic acid is catalysed by C3H. The enzyme used in this thesis was derived from *Saccharothrix espanaensis*, a gram-positive bacterium belonging to the order of *Actinomycetales* (Berner et al., 2006). In previous studies, C3H was successfully used in *E. coli* to produce caffeic acid, when *p*-coumaric acid was supplemented as a substrate (Rodrigues et al., 2015).

Caffeic acid *O*-methyltransferase (COMT)

Methyltransferases are essential for lignin formation in plant cell walls and are ubiquitous in plants (Hatfield and Vermerris, 2001). They are responsible for the production of various phenylpropanoids as the basis for the production of numerous polyphenols (Schönherr and Cernak, 2013). COMT is an enzyme participating in the phenylpropanoid pathway catalysing the *O*-methylation of different cinnamic acid derivatives (Inoue et al., 2000; Parvathi et al., 2001) like caffeic acid and 5-hydroxyferulic acid (Ferrer et al., 2008). A consequence of the low substrate specificity is the risk to produce several similar compounds, which are difficult to separate (Allewell, 2012; Yoon et al., 2012).

The methyltransferase from *Medicago sativa* (*M. sativa*) is a 43 kDa enzyme consisting of 365 amino acids and forms a dimer in the native stage. The availability of a crystal structure by X-ray crystallography (PDB ID: 1KYZ) provides information about the substrate binding site and the reaction mechanism (Zubieta et al., 2002) which is comparable to other plant-derived *O*-methyltransferases (Zubieta et al., 2001). The active site of the functional methyltransferase consists of two subunits. Each monomer comprises a catalytic C-terminal domain consisting of an α/β-Rossmann fold, which is needed to bind the cofactor *S*-adenosyl-L-methionine (SAM) (Rossmann et al., 1974).

At first, the hydroxyl group at the phenyl ring is deprotonated by a histidine at position 269. This allows the transmethylation of SAM and results in *S*-adenosyl-L-homocysteine (SAH) and the methylated substrate (Fig. 4).

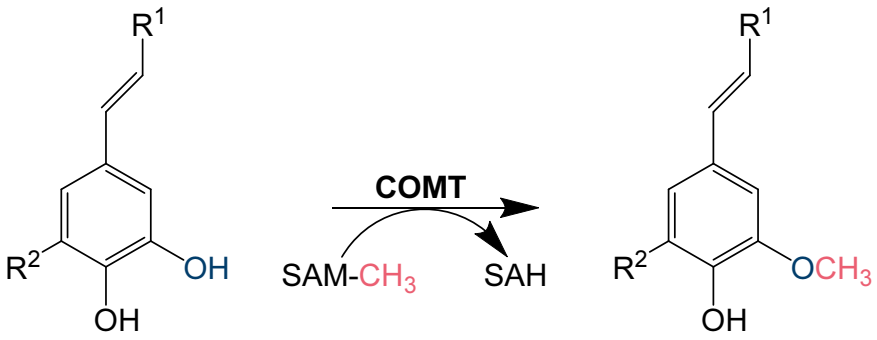

Fig. 4 Caffeic acid *O*-methyltransferase (COMT) catalyses the *O*-methylation of various phenylpropanoids. The hydroxyl group (blue) located in *meta*-position of the phenyl ring serves as methylation site, *S*-adenosyl-L-methionine (SAM) donates the methyl group (red) and is transformed to *S*-adenosyl-L-homocysteine (SAH). SAM: *S*-adenosyl-L-methionine, SAH: *S*-Adenosyl-L-homocysteine; R^1: -COOH (carboxylic acid), -CO (aldehyde), -COH (alcohol); R^2: -H (proton), -OCH_3 (*O*-methylation).

The non-specific activity of COMT towards several phenylpropanoids could be due to the relatively large substrate binding site next to His 269 and the cofactor (Zubieta et al., 2001).

1.3.3 Polyphenol synthesis: Multicopper oxidase/Laccase

Laccases are copper-containing enzymes and belong to the family of multicopper oxidases (Kües and Rühl, 2011). They use oxygen as co-substrate and the only by-product is water, which is the reason why they are described as “green catalysts”. Moreover, they are involved in the production of phenylpropanoid-derived polyphenols as they promote the oxidative coupling of two monolignols (Solomon et al., 1996). A single electron oxidation initiates the dimerization reaction through free radical coupling (Gang et al., 1999). These reactions are not stereochemically specific resulting in a variety of different lignans (Dixon, 2004) (Fig. 5).

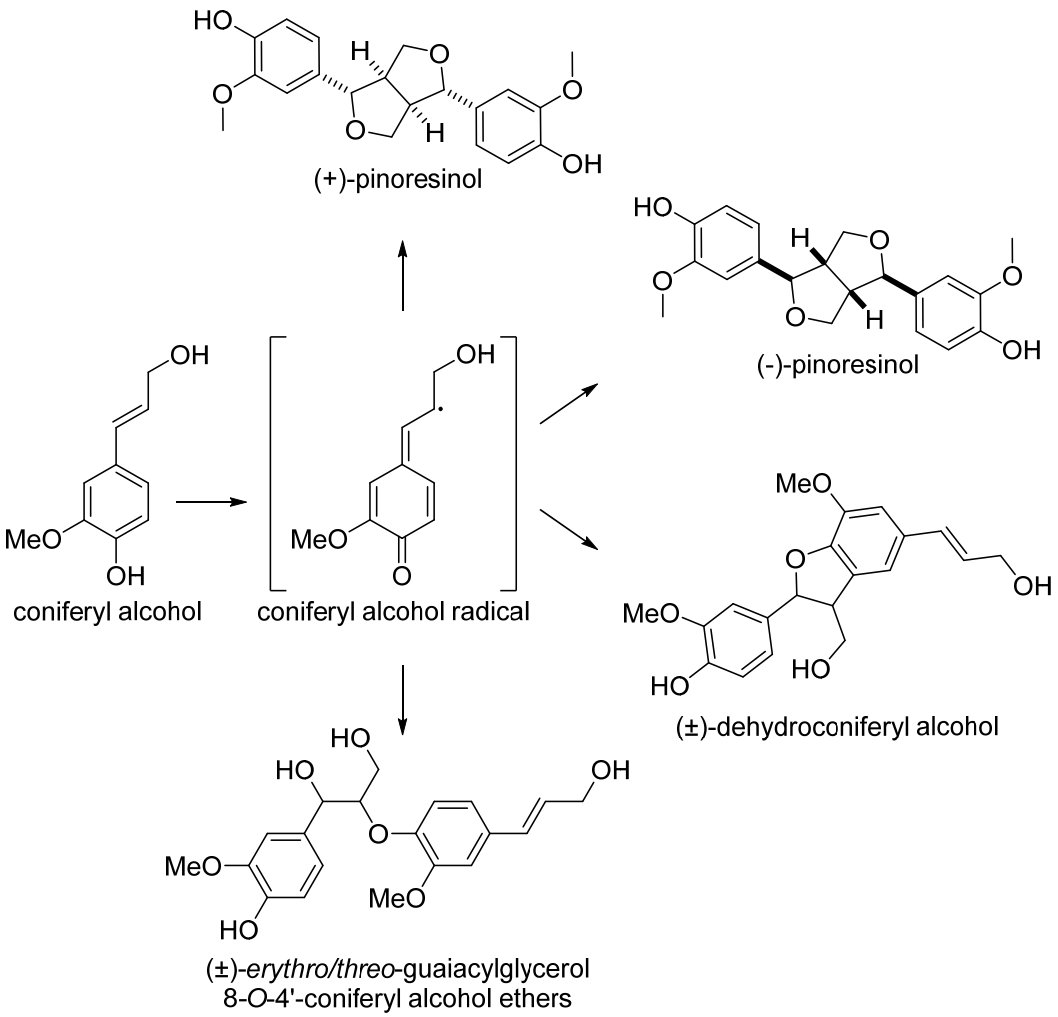

Fig. 5 Radical coupling products from coniferyl alcohol. Laccases produce radicals of coniferyl alcohol that allow for multiple forms of dimerization. Without the addition of dirigent proteins, the coupling is non-specific.

The multicopper oxidase CueO from *Rhodococcus erythropolis* was previously used for the *in vitro* lignan production and has a laccase activity (Classen et al., 2013). Multicopper oxidases are nearly ubiquitous in higher plants, fungi and bacteria and are known for their broad substrate spectrum. They participate in cell development, heavy metal resistance and in the formation and degradation of lignin (Hoegger et al., 2006). Amongst others, their application consists in the delignification of wood fibres, polymer synthesis or waste water cleaning in dyeing factories (Riva, 2006; Rodríguez et al., 1999).

1.4 Microbial production of phenylpropanoids and phenylpropanoid-derived polyphenols

(Milke, L., Aschenbrenner, J., Marienhagen, J., and Kallscheuer, N. 2018, Applied Microbiology and Biotechnology, cf. chapter 2.1)

Phenylpropanoids and phenylpropanoid-derived polyphenols are normally present in plants only in low concentrations, which impedes their extraction from their natural source (Georgiev et al., 2009). Furthermore, plants underly various environmental factors influencing the yield of desired compounds even more (Milder et al., 2005; Zhu et al., 2018). Another limitation is that plant tissues are composed of a mixture of phenylpropanoids with similar chemical properties, which makes the downstream processing time-consuming and costly (Chemler and Koffas, 2008). A different approach to produce such compounds is chemical synthesis, which, however, is laborious and time-consuming due to numerous synthetic steps including intermediate purifications (Maruyama et al., 1994; Pickel et al., 2010; Roy et al., 2002). A good alternative is the microbial production of phenylpropanoids and phenylpropanoid-derived polyphenols as available molecular biological tools enable the functional implementation of plant-derived pathways in the microbial metabolism (Marienhagen and Bott, 2013; Milke et al., 2018).

1.4.1 Phenylpropanoids

Cinnamic acid is “the simplest” phenylpropanoid and serves as chemical basis to produce additional phenylpropanoids. Its synthesis depends on the shikimate pathway, which provides the aromatic amino acids L-phenylalanine and L-tyrosine (Masuo et al., 2016). This pathway can be used in connection with a PAL for the production of cinnamic acid from glucose in *E. coli* (van Summeren-Wesenhagen and Marienhagen, 2015; Vargas-Tah et al., 2015). Another strategy is the addition of the direct precursor molecule L-phenylalanine to cultivations, which was performed using *Streptomyces lividans* and *E. coli* (Cui et al., 2014; Wang et al., 2015). The production of *p*-coumaric acid, caffeic acid, and ferulic acid was enabled by introducing genes encoding enzymes that catalyse these steps in *E. coli* (Berner et al., 2006; Choi et al., 2011; Rodrigues et al., 2015) (chapter 1.3.2). Further optimisation was achieved by an L-tyrosine overproducing *E. coli* strain resulting in higher product yields based on glucose (Kang et al., 2012; Lütke-Eversloh and Stephanopoulos,

2007; Pittard et al., 2005). The microbial production of 5-hydroxyferulic acid and sinapic acid was not described so far.

As mentioned before, enzymes participating in the monolignol pathway have a broad substrate range and were used for the production of various phenylpropanoids (Ferrer et al., 2008). An *E. coli* strain harbouring a phenylalanine ammonia lyase, a carboxylic acid reductase and an alcohol dehydrogenase was able to produce cinnamyl alcohol from a glycerol/glucose mixture (Klumbys et al., 2018). Moreover, another *E. coli* strain was engineered for *p*-coumaryl alcohol production and its productivity was enhanced by balancing gene expression of all pathway genes on the level of varying the spacing between the Shine-Dalgarno sequence and the START codon (Jansen et al., 2014; van Summeren-Wesenhagen et al., 2015). The production of caffeoyl alcohol and coniferyl alcohol was realised using immobilised cells or an optimised cultivation protocol with respect to the feeding strategy (Chen et al., 2017; Liu et al., 2017).

1.4.2 Polyphenols

In recent years, many microbial strains were constructed to produce polyphenols, especially flavonoids and stilbenes (Kaneko et al., 2003). For example the flavonoid naringenin was produced in *E. coli* by the heterologous expression of genes coding for a 4CL, chalcone synthase and chalcone isomerase (Hwang et al., 2003). These genes were used in *E. coli*, *C. glutamicum,* and *Saccharomyces cerevisiae* (*S. cerevisiae*) for naringenin production from different precursors like *p*-coumaric acid or L-tyrosine (Kallscheuer et al., 2016; Xu et al., 2011; Yan et al., 2005). The production was further optimised by the addition of cerulenin, which inhibits the fatty acid synthesis so that the stinted malonyl-CoA is then available for polyphenol synthesis (Santos et al., 2011; Wu et al., 2014). The same strategy was used to produce the stilbene pinosylvin from L-phenylalanine or cinnamic acid in *E. coli* and *C. glutamicum* (Kallscheuer et al., 2016; van Summeren-Wesenhagen and Marienhagen, 2015). The stilbene resveratrol was also successfully produced in *E. coli* (Lim et al., 2011), *C. glutamicum* (Kallscheuer et al., 2015; Kallscheuer et al., 2016; Kallscheuer et al., 2017), and *S. cerevisiae* (Li et al., 2016; Shin et al., 2011; Shin et al., 2012) from various precursors. Until now, microbial production of lignans was not described.

1.4.3 Production of non-natural compounds

Another promising alternative to natural polyphenols is the production of non-natural polyphenols. These analogous have pharmacological properties and have a toxic

effect on bacteria and fungi, but are not active against human cells (Cress et al., 2013; Fowler et al., 2011; Ramawat and Mérillon, 2013). On top of that, these compounds can help to get a deeper understanding in mechanisms of enzymatic catalysis and in requirements for substrate specificity (Bhan et al., 2015; Chen et al., 2007; Mora-Pale et al., 2013). The production of different non-natural flavanones and stilbenes with enzymes of a phenylpropanoid pathway was previously demonstrated (Chemler et al., 2007; Horinouchi, 2008; Horinouchi, 2009; Katsuyama et al., 2007b; Katsuyama et al., 2007a). The used pathway included a 4CL, which is also needed for monolignol synthesis (chapter 1.3.1). The synthesis was achieved by supplementing respective precursor molecules to the cultivation broth and the resulting product titres were comparable to those for natural compounds. This strategy is also called precursor directed synthesis (Pandey et al., 2016).

1.5 Aim of this thesis

This thesis focused on engineering of *E. coli* for the microbial production of phenylpropanoids and phenylpropanoid-derived polyphenols. This should contribute to improve the access and thus cover the high demand for such compounds in the pharmaceutical and food industries.

In part one the low substrate specificity observed with heterologous enzymes of the phenylpropanoid pathway was studied as strategy to produce various monolignols starting from respective cinnamic acid derivatives. All modifications introduced in these molecules by enzyme catalysis refer to the propene tail of the phenylpropanoids (Fig. 2). Furthermore, successfully produced monolignols with interesting substitutions at the phenyl ring for consecutive chemical reactions were subject to further improvements. This included optimisation regarding substrate supplementation and implementation of a suitable screening method to monitor further optimisations in cultivation conditions or strain development.

Another pathway involved in the phenylpropanoid synthesis mediates hydroxylation and *O*-methylation of the phenyl ring in phenylpropanoids (Fig. 3). The participating enzymes were examined to produce different cinnamic acid derivatives in part two. At first, a literature research was performed to evaluate suitable hydroxylases and *O*-methyltransferase mediating the substitutions at the phenyl ring. Enzymes catalysing these steps were used in whole cell biotransformation for the *in vivo* production of the desired cinnamic acid derivative. This is advantageous because the *O*-methylation of

caffeic acid and 5-hydroxyferulic acid can then be conducted without additional cofactor (SAM) supplementation (Fig. 3, Fig. 4). If necessary, the substrate range of this enzyme must be adopted to methylate caffeic acid and 5-hydroxyferulic acid leading to ferulic acid and sinapic acid, respectively. This was performed using site-saturation mutagenesis.

The third part focused on the *in vivo* lignan production with building blocks synthesised in part one with the phenylpropanoid pathway. For this purpose, a suitable enzyme had to be selected and was subject to initial optimisation of the expression system. Finally, lignan production was evaluated by supplementation of monolignols.

1.6 Key results

This thesis comprises in total three parts. The first part focusses on the monolignol pathway which is responsible for the reduction of various cinnamic acid derivatives to the corresponding monolignol (Fig. 6).

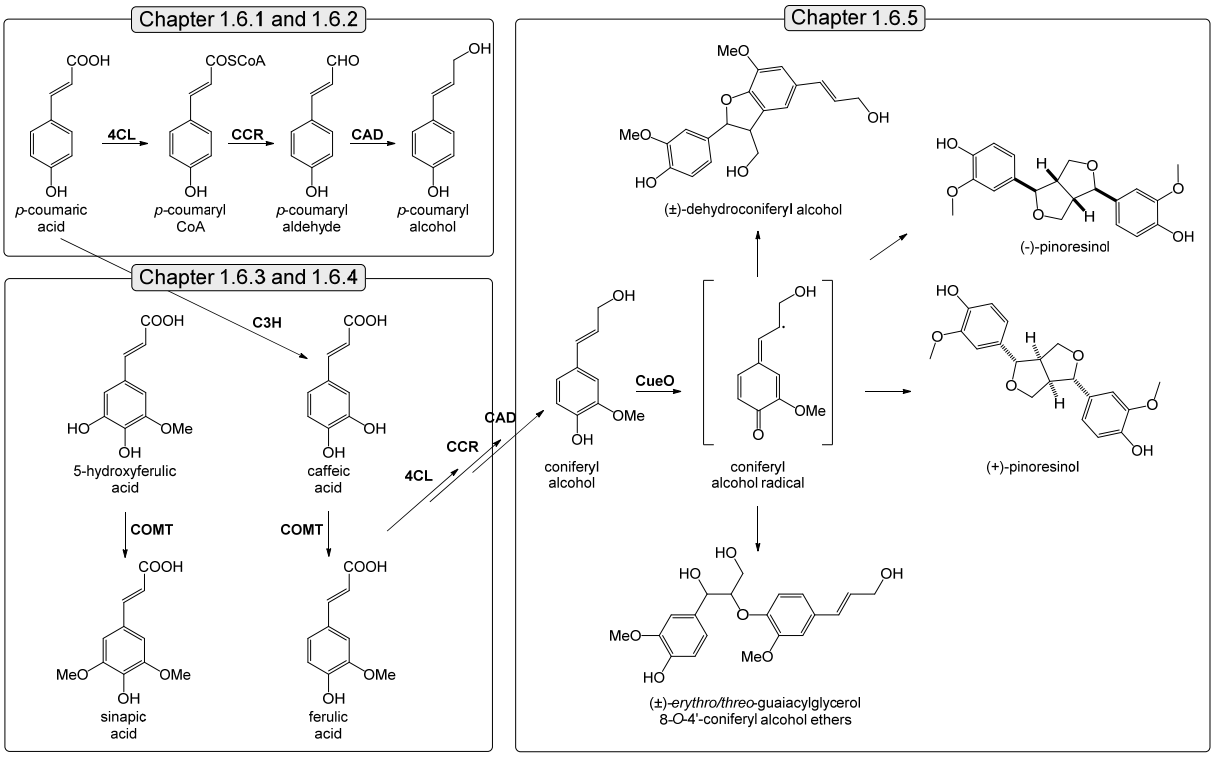

Fig. 6 General overview about the topics this thesis addresses. Chapters 1.6.1 and 1.6.2 focused the monolignol pathway from a cinnamic acid derivative to the corresponding monolignol (in this scheme demonstrated with *p*-coumaric acid and *p*-coumaryl alcohol). Chapters 1.6.3 and 1.6.4 addressed the production of various cinnamic acid derivatives. Pinoresinol production is described in chapter 1.6.5.

The part addresses the production of various natural and non-natural monolignols by an engineered *E. coli* strain (chapter 1.6.1). In addition, the optimisation of cultivation conditions regarding substrate supplementation and the development of a suitable screening method was focused (chapter 1.6.2).

The second part addresses the microbial production of cinnamic acid derivatives using other enzymes participating in the phenylpropanoid pathway (Fig. 6). This includes *E. coli* strains for the production of caffeic acid (chapter 1.6.3) and *E. coli* strains for the production of ferulic acid and sinapic acid (chapter 1.6.4).

The last part concentrated on the microbial lignan production from supplemented coniferyl alcohol (chapter 1.6.5).

1.6.1 Microbial production of natural and non-natural monolignols

(Aschenbrenner, J., Marx, P., Pietruszka, J. and Marienhagen, J. 2018, ChemBioChem, cf. chapter 2.2)

Enzymes participating in the phenylpropanoid pathway are known for their low substrate specificity, which is specifically advantageous as a broad range of different compounds can be converted with only one enzyme (Ferrer et al., 2008). However, this approach does only yield the desired target products in sufficient amount and purity, if the respective precursors are selectively supplied. In this thesis the catalytic versatility of the heterologous phenylpropanoid pathway to monolignols in *E. coli* was explored by supplementing different cinnamic acid derivatives as starting compounds to the cultivation medium (Aschenbrenner et al., 2018). The synthetic pathway towards the corresponding monolignols encompassed the following enzymes: tyrosine ammonia lyase (TAL) from the purple bacterium (*Rhodobacter sphaeroides*), 4-coumarate: CoA ligase (4CL) from parsley (*Petroselinum crispum*), and two enzymes derived from corn (*Zea mays*): cinnamyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) (Fig. 2). The genes were organised as synthetic operon under control of the IPTG-inducible T7 promoter. In a previous work, the genes were already functionally introduced in *E. coli* and the success of the strategy was shown by production of the monolignol *p*-coumaryl alcohol by cultivation of the strain in LB medium without any supplements (Jansen et al., 2014; van Summeren-Wesenhagen et al., 2015).

In a first step, the following naturally occurring cinnamic acid derivatives were supplemented to cultivations of this engineered *E. coli* strain to verify that the corresponding monolignols can be produced via the heterologous pathway: cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, hydroxyferulic acid, and sinapic acid (Fig. 7).

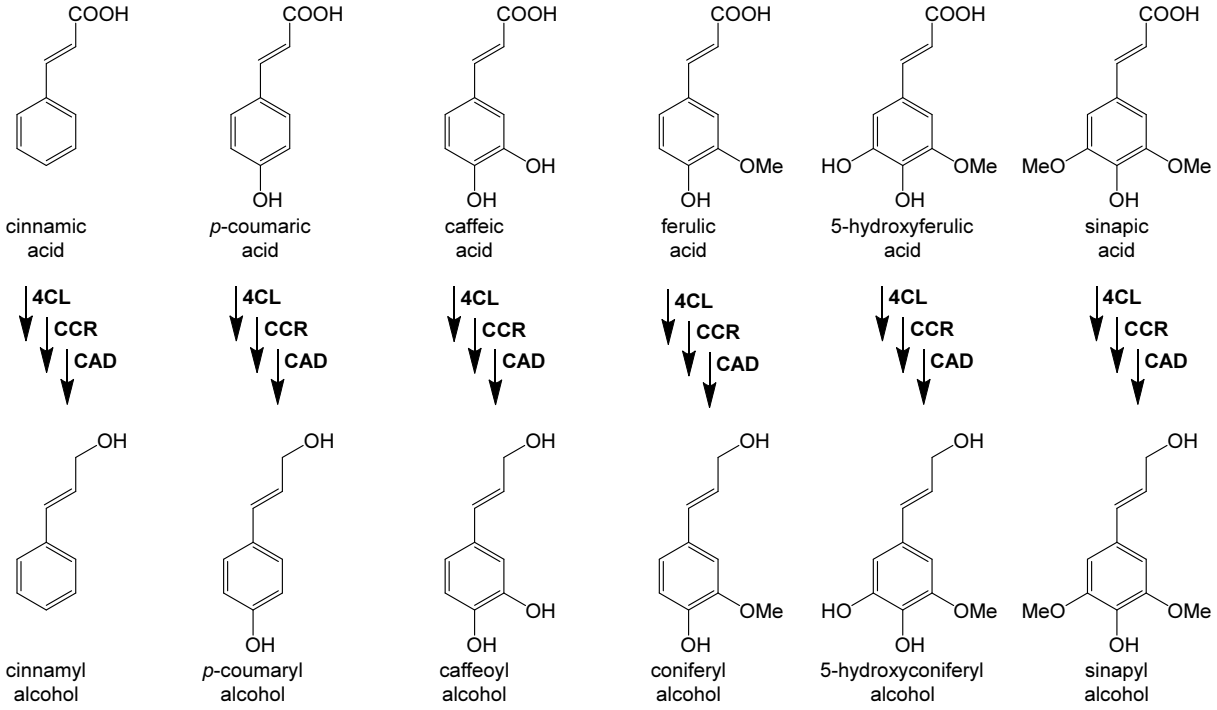

Fig. 7 Naturally occurring cinnamic acid derivatives (above) and the corresponding monolignols (below). 4CL: 4-coumarate: CoA ligase, CCR: cinnamyl-CoA reductase, CAD: cinnamyl alcohol dehydrogenase. The monolignol production was initiated by addition of the respective cinnamic acid derivative (2.5 mM) to the growing cells. After 17 h, the monolignol concentrations were determined by HPLC, respectively.

It turned out that the synthetic pathway can be used to reduce several naturally occurring cinnamic acid derivatives to the respective monolignols. Cinnamic acid is a phenylpropanoid with an unsubstituted phenyl ring and a precursor molecule for cinnamyl alcohol, which could be obtained with 195.4 mg/L (1.46 mM) using the engineered *E. coli* strain (chapter 2.2: Tab. 1). The production strain was also able to accumulate 121.8 mg/L (0.81 mM) *p*-coumaryl alcohol in culture supernatants with the same cultivation conditions (chapter 2.2). Moreover, the strain was also capable to synthesise 5.6 mg/L (0.03 mM) caffeoyl alcohol, which was the lowest concentration observed among all naturally occurring monolignols. The highest monolignol concentrations were detected during coniferyl alcohol production (327.8 mg/L,

1.82 mM) and was therefore subject to extractions of 1 L culture supernatant yielding 280 mg/L (1.55 mM). In addition, the production strain was also able to produce the two remaining monolignols, 5-hydroxyconiferyl alcohol (102.4 mg/L, 0.52 mM) and sinapyl alcohol (102.4 mg/L, 0.52 mM), which could be demonstrated for the first time.

In literature, the production of some monolignols were also addressed using different media, a different set of enzymes, a prolonged cultivation time, higher substrate concentrations or a different strategy regarding substrate supplementation (Chen et al., 2017; Klumbys et al., 2018; Liu et al., 2017). The main influence had the multiple substrate addition during biotransformations, because this strategy impeded the growth-inhibiting effect of cinnamic acid derivatives, which could be seen during this work and in previous studies (Matejczyk et al., 2017; Rodrigues et al., 2015). Nevertheless, this was the first time, that one *E. coli* strain was used to produce cinnamyl alcohol, *p*-coumaryl alcohol, caffeoyl alcohol, coniferyl alcohol, 5-hydroxyconiferal alcohol and sinapyl alcohol.

Motivated by these results for the production of natural monolignols, the synthesis of non-natural derivatives was also examined, which gives access to a variety of modifiable lignans (Kantchev et al., 2007). One of such substrates, which resembles sinapic acid, is the commercially available 3,4,5-trimethoxycinnamic acid with three *O*-methyl groups at the phenyl ring (Fig. 8).

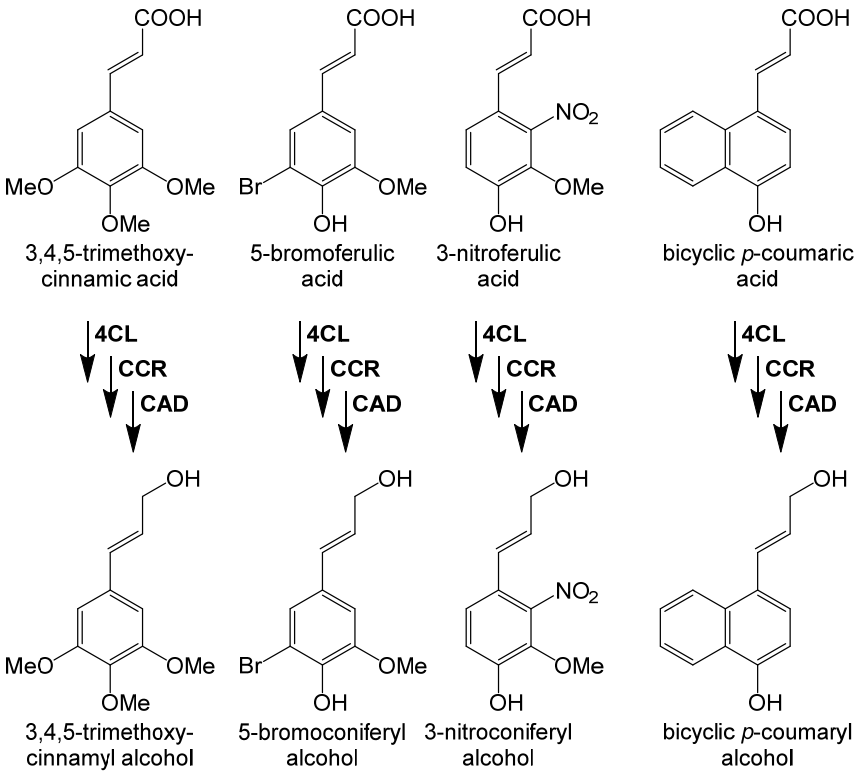

Fig. 8 Non-naturally occurring cinnamic acid derivatives (above) and the corresponding monolignols (below). 4CL: 4-coumarate: CoA ligase, CCR: cinnamyl-CoA reductase, CAD: cinnamyl alcohol dehydrogenase.

Nevertheless, only 4.5 mg/L (0.02 mM) 3,4,5-trimethoxycinnamyl alcohol, the corresponding monolignol, was detectable in culture supernatant (chapter 2.2: Tab. 1). 3-Nitroconiferyl alcohol was also successfully synthesised by the production strain (74.6 mg/L, 0.33 mM) and is of interest because of the nitro group on the phenyl ring. This reactive group enables additional chemical alterations of 3-nitroconiferyl alcohol-derived compounds (Kantchev et al., 2007). To further test the limits of the heterologous pathway, the sterically more challenging precursor bicyclic *p*-coumaric acid was subject to biotransformations. Astonishingly, 25.4 mg/L (0.13 mM) bicyclic *p*-coumaryl alcohol accumulated in the culture supernatant and seems to be a more suitable substrate for the monolignol pathway in contrast to caffeic acid. It should be stressed that qualitative NMR experiments revealed the accumulation of *p*-coumaryl aldehyde in the culture supernatant. This suggests that CAD, the last enzyme within the heterologous monolignol pathway, does not efficiently reduce this aldehyde compared to other cinnamyl aldehyde derivatives. 5-Bromoferulic acid was the best converted non-natural cinnamic acid derivative with 5-bromoconiferyl alcohol yielding the highest titres (462.2 mg/L, 1.78 mM) among all tested natural and non-natural

monolignols. This is beneficial since the bromine group allows for the consecutive chemical transformation of lignans (Kantchev et al., 2007).

This was the first time, that non-natural monolignols were produced with *E. coli*. Moreover, it is noteworthy, that the same engineered *E. coli* strain can be used to produce numerous natural and non-natural monolignols.

1.6.2 Development of a suitable screening method

(Aschenbrenner, J., Marx, P., Pietruszka, J. and Marienhagen, J. 2018, ChemBioChem, cf. chapter 2.2)

5-Bromoconiferyl alcohol production was chosen for further optimisation because of the high yield and the reactive bromine group which allows additional chemical transformations (Kantchev et al., 2007). As known from natural cinnamic acid derivatives, which have an inhibitory effect on microbial growth, an appropriate substrate concentration must be found to compromise product concentrations and microbial growth (Matejczyk et al., 2017; Rodrigues et al., 2015). For this purpose, *E. coli* cultivations were performed in 48-well microtiter plates in a microbioreactor system (Aschenbrenner et al., 2018). The cultivations were supplemented with varying concentrations of 5-bromoferulic acid (0 mM – 6 mM) and the backscatter was recorded for 17 h. The cultivations with substrate concentrations up to 3 mM revealed that the presence of 5-bromoferulic acid has a growth-inhibiting effect, as was found for natural phenylpropanoids in this study (chapter 2.2: Fig. 3). Substrate addition in concentrations exceeding 3 mM resulted in precipitation which impeded the determination of the culture backscatter over time (chapter 2.2). Moreover, the supplementation of these substrate concentrations did not lead to increased 5-bromoconiferyl alcohol concentrations, probably due to enhanced growth-inhibitory effect (Fig. 9). With respect to the 5-bromoconiferyl alcohol concentration, substrate concentrations around 2.5 mM turned out to be the most suitable since 225.7 mg/L (0.87 mM) were determined in culture supernatants (Fig. 9).

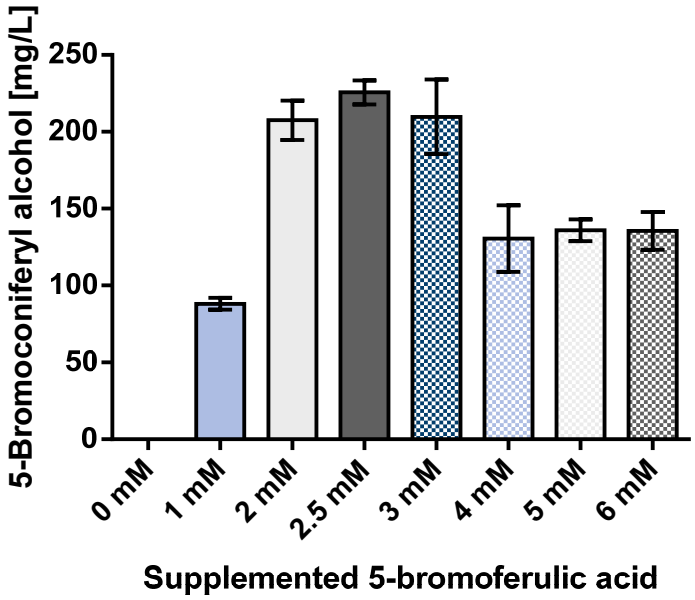

Fig. 9 Influence of different supplemented 5-bromoferulic acid concentrations on the production of 5-bromoconiferyl alcohol. *E. coli* was cultivated in 900 µL LB medium with different 5-bromoferulic acid concentrations in 48-well microtiter plates at 25 °C and 900 rpm. Heterologous gene expression was induced with 1 mM IPTG at the time of inoculation. 5-Bromoconiferyl alcohol concentration was determined with HPLC. Data represents average values and standard deviations from three biological replicates. For details see chapter 2.2, Fig. 3.

The growth-inhibiting effect of 5-bromoferulic acid could be circumvented by continuously feeding the substrate during biotransformations (Chen et al., 2017). One possibility, especially for large scale application, is fed-batch fermentation (Ezeji et al., 2004), where the substrate concentration is kept below the toxic level. For future small scale biotransformations a slow-release technique can be used to avoid growth-inhibiting effects caused by phenylpropanoid concentrations. This technique is based on a diffusion driven substrate release and consists of a feed reservoir system filled with a concentrated substrate solution, which diffuses through a dialysis membrane into the medium (Bähr et al., 2012; Jeude et al., 2006). Besides shake flasks cultivation this approach could also be used for biotransformation in microtiter plate scale (Wilming et al., 2014).

For optimisation of the strain and culture conditions, an appropriate screening system is required, which allows the fast evaluation of numerous clones. Noticeably, the culture supernatants at the end of biotransformations changed the colour, which is

most likely because of the produced monolignols (Fig. 10). This could be used for a fast, colorimetric screening method.

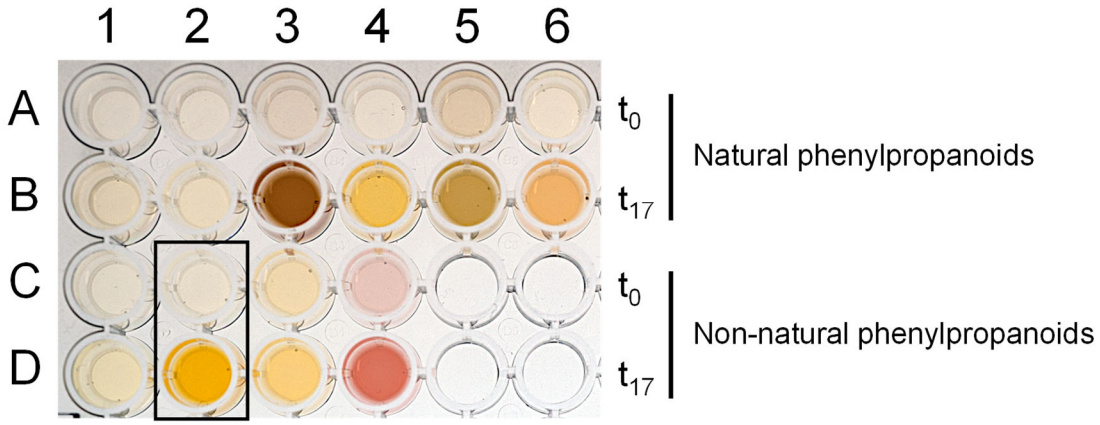

Fig. 10 Culture supernatants of different *E. coli* cultivations for monolignol production. *E. coli* harbouring the synthetic pathway for monolignol production was cultivated in 50 mL LB medium and 2.5 mM of the respective cinnamic acid derivatives were added. Samples were taken immediately after induction (t_0) and at the end of the biotransformation (t_{17}). The supernatants were obtained by centrifugation. After induction, only the cinnamic acid derivative could be found in culture supernatants. At the end of cultivation, cinnamyl alcohol derivatives were detectable. A1: cinnamic acid, B1: cinnamyl alcohol, A2: *p*-coumaric acid, B2: p-coumaryl alcohol, A3: caffeic acid, B3: caffeoyl alcohol, A4: ferulic acid, B4: coniferyl alcohol, A5: 5-hydroxyferulic acid, B5: 5-hydroxyconiferyl alcohol, A6: sinapic acid, B6: sinapyl alcohol, C1: 3,4,5-trimethoxycinnamic acid, D1: 3,4,5-trimethoxycinnamyl alcohol, C2: 5-bromoferulic acid, D2: 5-bromoconiferyl alcohol, C3: 3-nitroferulic acid, D3: 3-nitroconiferyl alcohol , C4: bicyclic *p*-coumaric acid, D4: bicyclic *p*-coumaryl alcohol. The most interesting compound is framed.

In this thesis the production of 5-bromoconiferyl alcohol was focused. Absorbance measurements of the respective supernatants (Fig. 10, C2, D2) resulted in 470 nm as the best detection wavelength to monitor product formation. This characteristic could be used in the future as a fast colorimetric screening method for strains with enhanced 5-bromoconiferyl alcohol production.

1.6.3 Combinatorial biosynthesis using 4-coumarate 3-hydroxylase (C3H)

Hydroxylation of the phenyl group gives access to various cinnamic acid derivatives, which can be used as precursors for monolignol synthesis (Fig. 6). The functional integration of the catalysing enzyme in the monolignol pathway would enable the synthesis of different monolignols, although only one cinnamic acid derivative is used as substrate in biotransformations. The hydroxylation is performed with C3H, which catalyses the conversion of *p*-coumaric acid to caffeic acid (Fig. 3). In this thesis, C3H

derived from *Saccharothrix espanaensis* was used (Berner et al., 2006), as it was previously successfully used to produce caffeic acid in *E. coli* (Rodrigues et al., 2015).

To test the functionality of the heterologously expressed C3H in *E. coli*, biotransformations were supplemented with 2.5 mM (410 mg/L) *p*-coumaric acid and culture supernatants were analysed with HPLC for phenylpropanoid concentrations. At the end of biotransformations, 0.12 mM (22 mg/L) caffeic acid was produced. This proves that the engineered strain is capable of producing caffeic acid from *p*-coumaric acid, although the conversion is relatively low. As a next step it was tested whether this enzyme can be combined to the monolignol pathway to produce caffeoyl alcohol from *p*-coumaric acid. For this purpose, *c3h* was introduced into the pathway responsible for monolignol production (chapter 1.6.1, Fig. 2). For the subsequent cultivation, two products were possible. Either *p*-coumaric acid will be converted directly to *p*-coumaryl alcohol or *p*-coumaric acid will be converted to caffeic acid and subsequently to caffeoyl alcohol (Fig. 11).

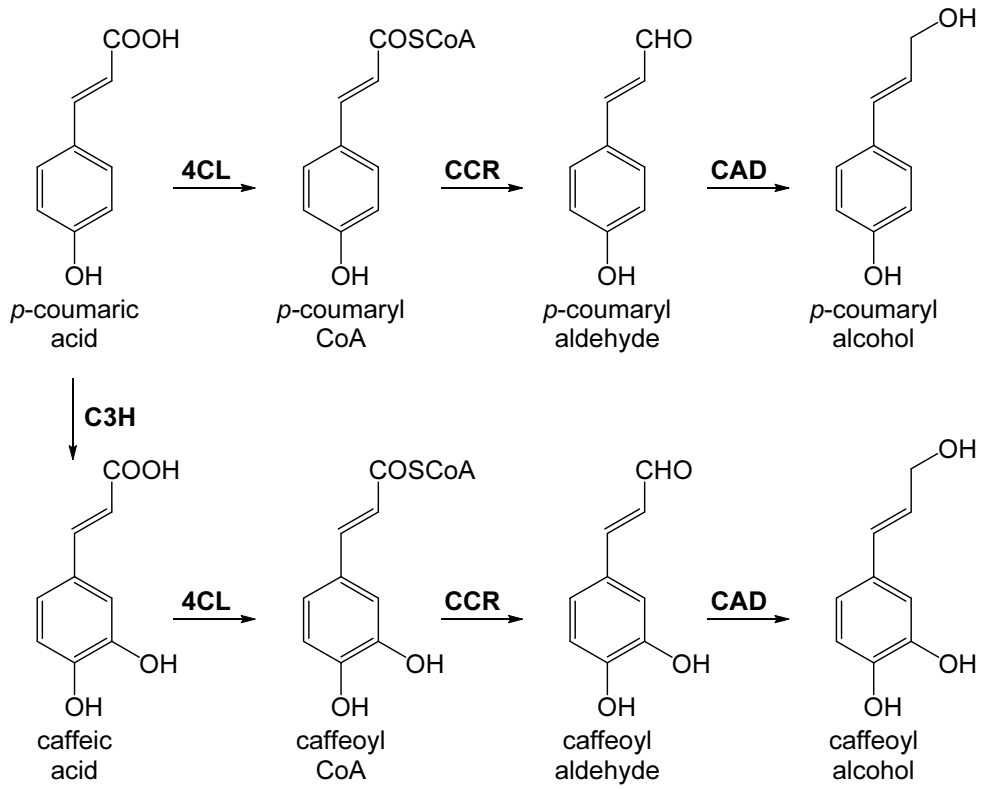

Fig. 11 Pathway from *p*-coumaric acid to *p*-coumaryl alcohol and caffeoyl alcohol. C3H: 4-coumarate 3-hydroxylase, 4CL: 4-coumarate: CoA ligase, CCR: cinnamyl-CoA reductase, CAD: cinnamyl alcohol dehydrogenase.

Biotransformations were supplemented with 2.5 mM *p*-coumaric acid and LC/MS analysis had to be used for sample measurement, because no caffeoyl alcohol reference material was available at that time. All four compounds were identified in culture supernatants: *p*-coumaric acid, caffeic acid, *p*-coumaryl alcohol and caffeoyl alcohol. The results confirmed the successful combination of an enzyme responsible for the modification of the phenyl group with enzymes responsible for reduction of the propene tail for the combinatorial biosynthesis of different monolignols in *E. coli*. However, it demonstrated that the promiscuous enzymes can not be used for the selective monolignol production, when multiple precursor molecules are present in the cultivation broth.

1.6.4 *In vivo* application and engineering of caffeic acid *O*-methyltransferase (COMT)

(see chapter 2.3, Engineering the substrate specificity of a caffeic acid *O*-*methyltransferase* from *Medicago sativa*)

As already pointed out (chapter 1.3.2), COMT belongs to the family of transferases and participates in the phenylpropanoid pathway. It catalyses the methylation of different phenylpropanoids, like caffeic acid and 5-hydroxyferulic acid (Ferrer et al., 2008; Inoue et al., 2000; Parvathi et al., 2001).

In this thesis COMT from *M. sativa* was selected to mediate *O*-methylation because this enzyme reached the highest product yields in literature (Wang et al., 2015) and because of its capability to convert caffeic acid and 5-hydroxyferulic acid (Zubieta et al., 2002) (Fig. 6). Since the SAM cofactor regeneration is ATP-dependent (Markham et al., 1980), the application of SAM-dependent enzymes *in vitro* is limited. Furthermore, the cofactor SAM is instable and expensive and the side product SAH is an inhibitor for many methyltransferases (James et al., 2002). Whereas one possible solution is the implementation of an *in vitro* SAM regeneration system (Mordhorst et al., 2017), whole cell biotransformations, as performed in this thesis, could overcome these limitations much easier, due to the internal regeneration of ATP in the living cell. However, the low substrate specificity of this enzyme leads to the production of several similar compounds, which are difficult to separate (Allewell, 2012; Yoon et al., 2012). Site-saturation mutagenesis was used to enhance the production of ferulic acid and sinapic acid and to alter the substrate specificity.

For this purpose, the constructed libraries consisted of mutants with alterations in the amino acid sequence at positions 135, 136, 162, and 172 (Fig. 12).

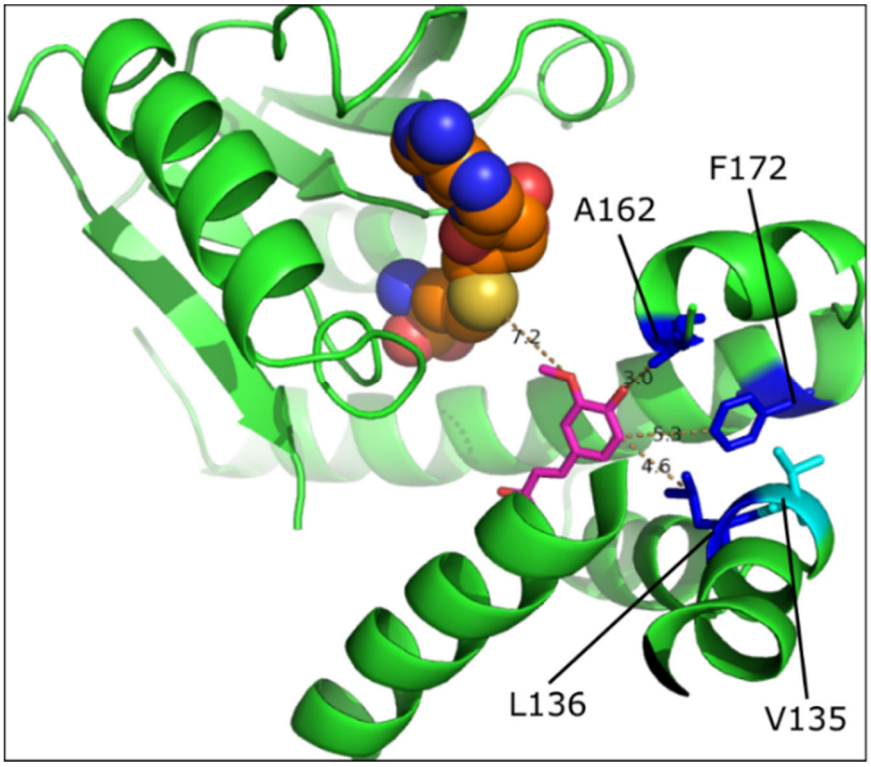

Fig. 12 Active centre of COMT. *S*-Adenosyl-L-homocysteine (SAH) is shown as space-filling structure (yellow: sulphur, red: oxygen, blue: nitrogen). Ferulic acid, the product of the *O*-methylation, is presented in magenta. Amino acid residues with postulated impact on the substrate specificity and which were subject to site-saturation mutagenesis are coloured in blue. Dashed lines assign the distance in Å. This image was produced with PyMOL (PDB ID: 1KYZ) and was taken from the Master thesis of Sascha Jansen (Jansen, 2017). For details see chapter 2.3, Fig. 4.

These positions were focused and were target sites for site-saturation mutagenesis, because an available crystal structure of COMT derived from *M. sativa* revealed that the side chains of L136, A162 and F172 are responsible for binding of not substituted substrate or *O*-methylated substrate in *meta*-position (Bugos et al., 1992; Inoue et al., 2000; Parvathi et al., 2001; Zubieta et al., 2002). Moreover, a nucleotide sequence alignment of a different *O*-methyltransferase with higher specificity towards caffeic acid revealed instead of substitutions in position 136, 162 and 172, a substitution of V135 to isoleucine and could cause the altered substrate specificity (Wiens and Luca, 2016).

The simultaneous saturation of multiple codons has the advantage that synergistic effects can be elucidated, which might have a positive effect on product formation

(Shivange et al., 2009). In a first step, some clones of the constructed library were examined regarding amino acid diversity in the targeted positions. Nucleotide sequencing of *comt* revealed at least one nucleotide substitution in each codon causing an amino acid variation at each position and multiple substitutions in one variant.

Detailed information about the screening set-up, which was performed in 96-deep well plates, can be found in chapter 2.3. In total, 1080 COMT variants with substitutions in position 136, 162 and 172 were tested regarding ferulic acid or sinapic acid concentration. One third of all tested variants were inactive, two third produced exclusively sinapic acid and 4 % can produce both phenylpropanoids. Based on this data, strains with promising COMT variants were selected and used in shake flask scale (10 mL) for further characterization. The *comt* sequences of these variants were also examined to get information about amino acid substitutions. It could be elucidated, that amino acid substitution at the selected position influenced productivity and substrate specificity. As demonstrated in Fig. 13, strains containing the wild type COMT produced 37.3 mg/L (0.19 mM) ferulic acid and 53.8 mg/L (0.24 mM) sinapic acid.

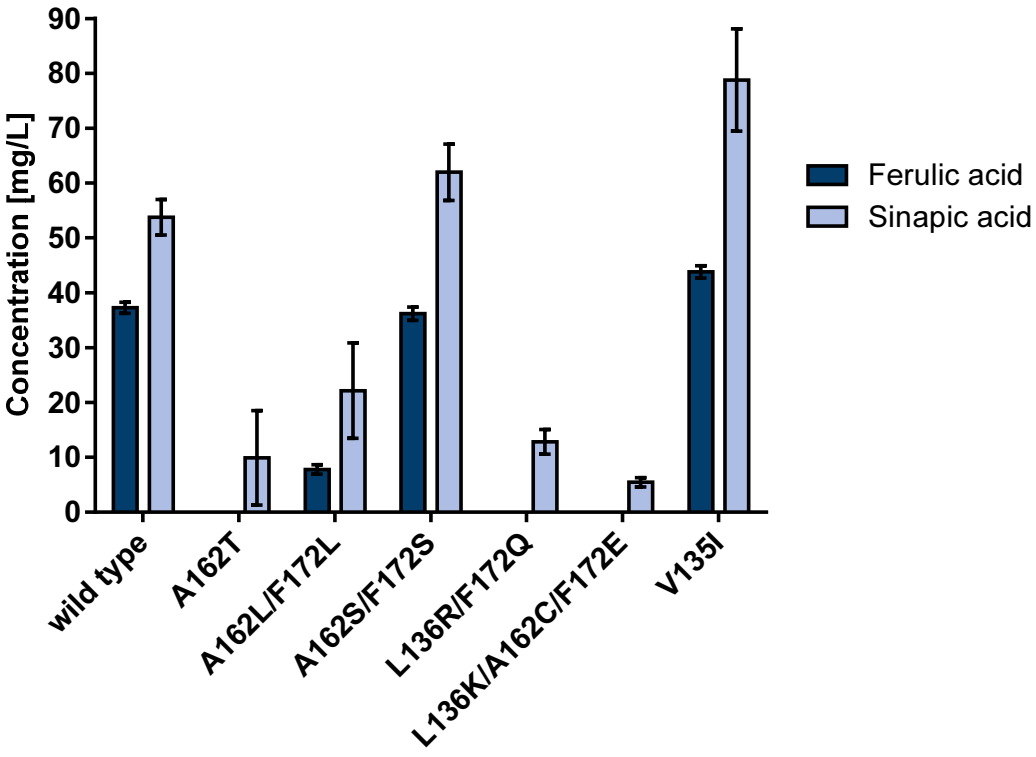

Fig. 13 Ferulic acid and sinapic acid production in different *E. coli* strains expressing various COMT variants. Strains were cultivated in 10 mL TRIS/HCl buffered YNB medium in 100 mL baffled shake flasks at 25 °C and 130 rpm. Heterologous gene expression was induced with 1 mM IPTG at OD_{600} 0.6 and caffeic acid or 5-hydroxyferulic acid was added to a

final concentration of 2.5 mM. Ferulic acid and sinapic acid were determined by HPLC. Data represent average values and standard deviations from three biological replicates. Results were taken from the Master thesis of Sascha Jansen (Jansen, 2017).

An exclusively sinapic acid production was achieved by three strains, which contained COMT variants with amino acid substitutions in position 136 to arginine or lysine, in position 162 to threonine or cysteine and in position 172 to glutamic acid and glutamine (Fig. 13). Substitutions in position 162 and 172 to serine resulted in ferulic acid concentrations comparable to the wild type and an increase of 15 % for sinapic acid production. As expected, the productivity is reduced with the quantity of amino acid substitutions introduced into COMT. A strain harbouring the substitution V135I (Wiens and Luca, 2016) resulted in 43.8 mg/L (0.23 mM) ferulic acid and 78.8 mg/L (0.35 mM) sinapic acid, which is an increase of 17 % and 46 % compared to the wild type. A direct impact of this amino acid substitution on the substrate binding pocket formation is unlikely, because the residue is not in direct proximity to the substrate (Fig. 12). However, the folding of the α-helix, which is adjacent to the substrate binding site and includes the residue at position 136, could be affected by a substitution at position 135 (Fig. 12). Similar second shell interactions have already been described earlier (Lingen et al., 2002).

The previously found COMT variants A162S/F172S and L136R/F172Q were subject to site-saturation mutagenesis at position 135 to increase the product titres or alter the substrate specificity of whole cell biotransformations. Variant A162S/F172S was selected due to increased ferulic acid production and L136R/F172Q due to selective sinapic acid production (Fig. 11). In contrast to the first screening approach, the site-saturation of one codon in these sub-libraries result in only 32 possible gene variants coding for 20 amino acids. Consequently, screening in only one 96-deep well plate was sufficient to cover more than 95 % of all possible variants (Firth and Patrick, 2008). Under screening conditions, 14 variants produced exclusively ferulic acid and 29 variants produced exclusively sinapic acid. Nucleotide sequencing of *comt* revealed substitution at position 135 to threonine, arginine, serine, glycine, glutamine and leucine.

All observed amino acid substitutions in position 135, 136, 162 and 172 differ in their size and in their functional groups. This includes polar amino acids (S, T, Q), positive

charged amino acids (K, R) and negatively charged amino acids (E). These characteristics contribute to substrate specificity (Betts and Russell, 2007; Schneider et al., 2003), but the exact interactions are difficult to define. Substitutions in positions located directly in the substrate binding pocket (position 136, 162 and 172) led to reduced activity rather than substitutions in positions indirectly related to the binding pocket (position 135)(Fig. 12, Fig. 13). This suggested that maybe substitutions at different positions should be taken in considerations.

The microtiter plate-based screening limited the selection of variants, because only few variants were found with increased ferulic acid production. A high-throughput screening approach, for example fluorescence-activated cell sorting (FACS) in connection with biosensors, could help to screen 70,000 variants within seconds to overcome these limitations (Marienhagen and Bott, 2013; Yang and Withers, 2009).

Strains selected from the screening in 96-deep well plates were additionally subjected to phenylpropanoid production in shake flasks. In principle, cultivations in shake flasks yielded higher product concentrations and a different substrate specificity than cultivations in deep well plates. The approaches differed in culture treatment as gene expression in shake flasks was induced at OD_{600} 0.6, whereas the expression in deep well plates was induced directly from the beginning. As a consequence, strains cultivated in deep well plates needed energy for the synthesis of recombinant protein and this energy is lacking for biomass production causing a longer lag phase at the beginning of the cultivation and a reduced phenylpropanoid production (Donovan et al., 1996). Furthermore, unfavourable cultivation parameters like low shaking frequencies and high filling volumes have an effect on the oxygen transfer rate, the cell growth and as a result the productivity of cultivations (Losen et al., 2004; Zimmermann et al., 2006). These parameters differ hugely between cultivations in microtiter plates and shake flasks and hamper the comparability between product yields and substrate specificity under screening and under production conditions (Duetz, 2007). The cultivation conditions should therefore be adjusted (Reetz and Carballeira, 2007). As a consequence, the gained knowledge can contribute to even higher ferulic acid and sinapic acid concentrations due to optimised cultivation conditions in shake flasks (Wewetzer et al., 2015).

1.6.5 Expression of *cueO* and *in vivo* pinoresinol synthesis

One goal of this thesis was the microbial production of lignans (Fig. 6). As mentioned in chapter 1.1 this polyphenol has a positive effect on health, but the microbial production was not described yet (chapter 1.4.2).

The multicopper oxidase CueO from *Rhodococcus erythropolis* catalyses lignan synthesis and was previously used for the lignan production *in vitro* (Classen et al., 2013)[1]. The gene *cueO* was expressed in *E. coli* under the control of a T7 expression system and the resulting protein pattern was analysed by an SDS-PAGE analysis (Fig. 14, A).

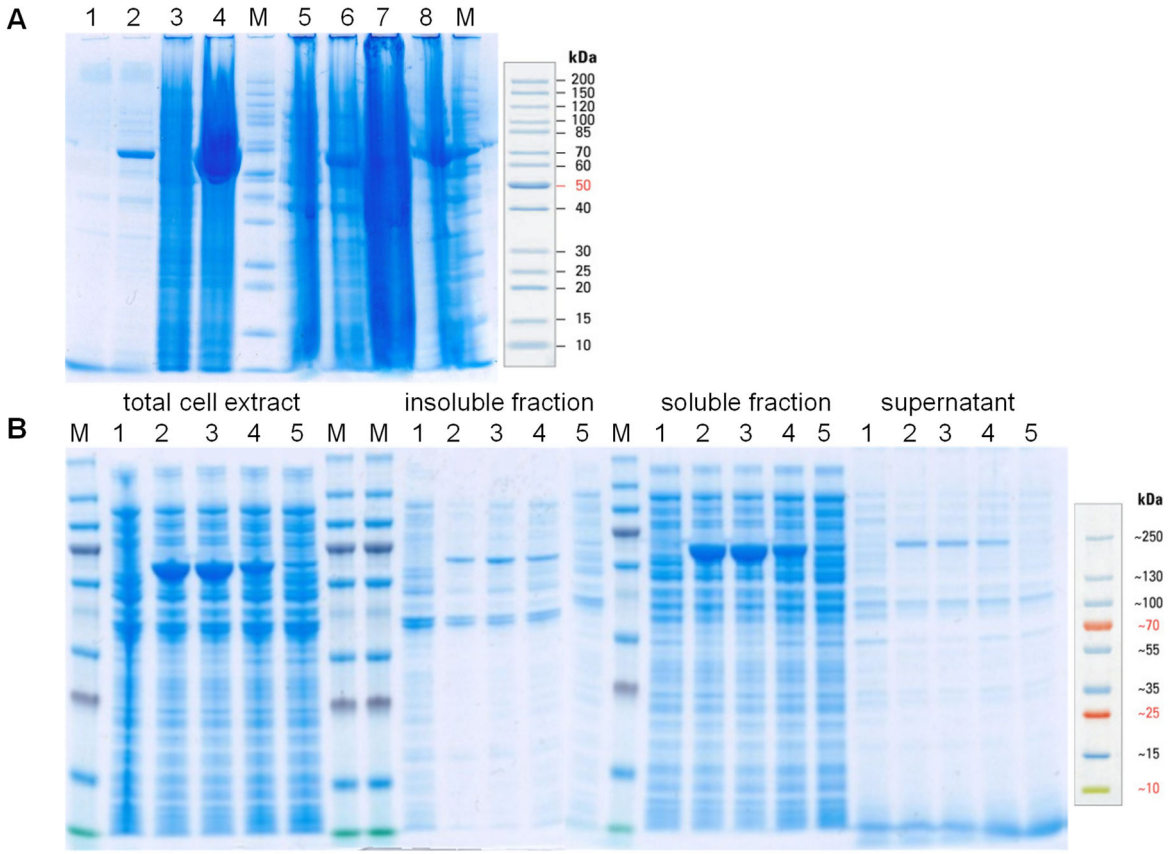

Fig. 14 CueO production in different *E. coli* strains. (A) *E. coli* harbouring pHT-*cueO* to produce CueO (54.7 kDa) under control of the T7 expression system was cultivated in 500 mL TB medium in a baffled 5 L Erlenmeyer flask. Cultivation was induced after reaching OD_{600} 0.6 with 1 mM IPTG and was incubated for 16 h at 25°C and 130 rpm. Odd numbers: negative control strain (*E. coli* pHT), even numbers: production strain (*E. coli* pHT-*cueO*).1-2: supernatant, 3-4: total cell extract, 5-6: insoluble fraction of total cell extract, 7-8: soluble fraction of total cell extract. (B) *E. coli* DH10B harbouring pBAD-*cueO* to produce CueO (54.7 kDa) under control of the pBAD expression system was cultivated in 50 mL TB medium in a baffled 500 mL Erlenmeyer flask. Cultivation was induced after reaching OD_{600} 0.6 with

[1] The gene was kindly provided by Dr. Thomas Classen, Institute of Bio- and Geosciences, IBG-1: Bioorganic Chemistry

different arabinose concentrations and was incubated for 16 h at 25°C and 130 rpm. 1: negative control strain (*E. coli* DH10B pBAD), 2-5: production strain (*E. coli* DH10B pBAD-*cueO*). 1, 2: 0.2 % arabinose, 3: 0.02 % arabinose, 4: 0.002 % arabinose, 5: 0.0002 % arabinose.

The results in Fig. 14 demonstrate that CueO (54.7 kDa) was successfully produced in the soluble fraction of the constructed strain. Besides the previous published expression protocol (cultivation in TB medium and 10 mM copper sulphate), the production was also tested in LB medium and 10 mM copper sulphate. This approach was not successful, because the cells were not able to grow in the unbuffered medium.

The focus within this thesis was the *in vivo* lignan production starting from supplemented monolignol (Fig. 6). For the connection of lignan production with the previous implemented monolignol production (chapter 1.6.1, chapter 2.2), a tight gene regulation is necessary. This enables the production of lignans from monolignols instead of production already from precursor molecules like cinnamic acid derivatives, which can also serve as substrate in this reaction (Gunne and Urlacher, 2012; Jin et al., 2010). Moreover, the oxidative coupling catalysed by CueO and the required copper addition to cultivation broth is harmful to the microorganism (Galli et al., 2004) and would have a negative effect on simultaneous monolignol production. Therefore, a strain must be capable to reduce cinnamic acid derivatives to the respective monolignols as a first step and then produce an enzyme for the oxidative coupling of two monolignols for lignan formation. The pBAD expression system allows a tightly controlled regulation of a target gene *in vivo* and can be induced with arabinose (Guzman et al., 1995). Moreover, it shows only a low expression when the promoter is not induced (Balzer et al., 2013). The strain *E. coli* DH10B harbouring pBAD-*cueO* was used to produce CueO. It lacks the *leuLABCD* operon (part of Δ(*ara leu*)*7697*) and can therefore be used for the induction with arabinose (Durfee et al., 2008). The strain was induced with various arabinose concentrations (0.0002 % - 0.2 %) and CueO production was analysed by SDS-PAGE (Fig. 14, B). CueO could be found in the supernatant and in the total cell extract. Furthermore, it was primarily present in the soluble fraction of the total cell extract than in the insoluble fraction. From this it could be concluded, that CueO exists rather in a dissolved form than in (probably inactive) inclusion bodies. The protein amount decreases with lower arabinose concentrations and was not detectable when the strain was induced with 0.0002 % arabinose. This

tight gene regulation of *cueO* could be used in the future to connect the microbial monolignol production under control of the T7 expression system (chapter 1.6.1, chapter 2.2), with the subsequent lignan production under control of the pBAD expression system reducing the previously described harmful effects.

The next step was to test whether an *E. coli* strain producing CueO is able to catalyse the reaction from coniferyl alcohol to (+)-pinoresinol. For this purpose, the production strain was cultivated in the presence of 1 mM coniferyl alcohol to examine the *in vivo* (+)-pinoresinol formation in three biological replicates. After 17 h biotransformation, 0.17 mM (60.8 mg/L) (+)-pinoresinol was detectable in the culture supernatant using HPLC[2]. This was the first time that microbial (+)-pinoresinol production was observed.

1.7 Conclusion and Outlook

The results obtained in this thesis show that *E. coli* strains equipped with enzymes participating in the phenylpropanoid pathway are capable to produce numerous phenylpropanoids including cinnamic acid derivatives and monolignols. Moreover, an engineered *E. coli* strain is also able to produce the polyphenol (+)-pinoresinol, which is related to the class of lignans (Fig. 6).

In this context it was demonstrated that an *E. coli* strain harbouring enzymes of the synthetic monolignol pathway represents a suitable whole cell biocatalyst for the production of natural and non-natural occurring monolignols, thereby giving access to chemically interesting and alterable polyphenols (chapter 1.6.1, chapter 2.2). This was achieved using enzymes with low substrate specificity resulting in the acceptance of a broad range of phenylpropanoid-like compounds. Among other produced monolignols, the microbial production of 5-hydroxyconiferyl alcohol and sinapyl alcohol as well as the microbial production of non-natural monolignols was demonstrated for the first time. Engineering the heterologous enzymes regarding productivity could lead to even higher product concentrations and could benefit from the screening method elaborated in this thesis (chapter 1.6.2, chapter 2.2).

Besides reaction engineering could further increase the monolignol yield. For example, a fed-batch system could reduce inhibitory effects caused by the substrates and improve product formation as previously discussed (chapter 1.6.2).

[2] HPLC-measurements were carried out by Patrick Marx, Institute of Bio- and Geosciences, IBG-1: Bioorganic Chemistry

The second part addressed the production of various cinnamic acid derivatives (Fig. 6). At first, a 4-coumarate 3-hydroxylase was successfully used to produce caffeic acid from *p*-coumaric acid *in vivo* (chapter 1.6.3). In combination with the previously used monolignol pathway, the constructed *E. coli* strain was able to produce caffeoyl alcohol and *p*-coumaryl alcohol from *p*-coumaric acid (chapter 1.6.3). This demonstrated the promiscuity of the synthetic pathway stressing the challenge of the broad substrate spectrum regarding selective monolignol production. Moreover, a caffeic acid *O*-methyltransferase was engineered for increased methylation of caffeic acid and 5-hydroxyferulic acid in whole cell biotransformations (chapter 1.6.4, chapter 2.3). It was revealed that amino acid substitutions in four positions located in or adjacent to the methoxy binding pocket, increased the productivity and changed the substrate specificity towards caffeic acid and 5-hydroxyferulic acid. These variants can contribute to a better understanding of these enzymes and might prove useful for the metabolic engineering of microbes for the biotechnological production of more complex phenylpropanoid-derived compounds, in future.

Screening of the remaining variants could open the possibility to find variants with different amino acid substitutions causing higher product yields or an enhanced substrate specificity. Moreover, substitutions in other positions adjacent to the binding pocket could also be promising as discussed previously (chapter 1.6.4). For a better comparability of screening and production results, the cultivation conditions should be adjusted. The gained knowledge could also contribute to higher yields in large scale set-up (chapter 1.6.4, chapter 2.3).

A further result of this thesis was the successfully production of an enzyme needed for lignan synthesis in *E. coli* under control of different gene expression systems and optimal induction conditions (chapter 1.6.5). The examination of the tight pBAD expression system would allow the connection of the previously implemented monolignol pathway and the enzyme mediating lignan production without the previously described drawbacks (chapter 1.6.5). Furthermore, the *in vivo* production of (+)-pinoresinol was demonstrated in *E. coli* for the first time using the constructed production strain.

Additional endeavours should be directed towards production of further lignans by supplementation of various monolignols. In order to quantify lignan production, huge efforts must be put into the development of analytical methods since the lack of suitable

dirigent proteins lead to non-specific production and numerous lignans are synthesised (chapter 1.3.3).

To conclude, *E. coli* is a suitable host to produce different phenylpropanoids like cinnamic acid derivates and monolignols, but is also capable to produce lignan, a plant-derived polyphenol. Further engineering of participating enzymes and implementation of large-scale production is required to increase the concentrations of desired compounds.

2 Publications and Manuscripts

2.1 Production of plant-derived polyphenols in microorganisms: current state and perspectives

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MINI-REVIEW

Production of plant-derived polyphenols in microorganisms: current state and perspectives

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Abstract
Plants synthesize several thousand different polyphenols of which many have the potential to aid in preventing or treating cancer, cardiovascular, and neurodegenerative diseases. However, plants usually contain complex polyphenol mixtures impeding access to individual compounds in larger quantities. In contrast, functional integration of biosynthetic plant polyphenol pathways into microorganisms allows for the production of individual polyphenols as chemically distinct compounds, which can be synthesized in large amounts and can be more easily isolated. Over the last decade, microbial synthesis of many plant polyphenols could be achieved, and along the way, many decisive bottlenecks in the endogenous microbial host metabolism as well as in the heterologous plant pathways could be identified. In this review, we present recent advancements in metabolic engineering of microorganisms for the production of plant polyphenols and discuss how current challenges could be addressed in the future.

Keywords Polyphenols · Metabolic engineering · Phenylpropanoids · Resveratrol · Naringenin · Malonyl-CoA

Introduction

In addition to alkaloids and isoprenoids, polyphenols constitute the third class of plant secondary metabolites (Bourgaud et al. 2001). By definition, polyphenols are characterized by two or more aromatic rings and at least two phenolic hydroxy groups, but a few hydroxylated one-ring aromatics such as gallic acid or pyrogallol have been also added to this group (Badhani et al. 2015; Tinh et al. 2016). Typically, polyphenolic compounds are not directly involved in plant growth and propagation. Instead, they counteract microbial infections or are involved in protecting the plant against UV radiation, i.e., by neutralizing reactive oxygen species in light-exposed plant tissues (Bennett and Wallsgrove 1994; Kootstra 1994). Furthermore, polyphenols can also confer coloration, attract pollinators, or provide protection from herbivores (Crozier et al. 2006; Holton and Cornish 1995).

Due to their natural function as antioxidants and radical-scavenging agents, some polyphenols demonstrate health-promoting effects in humans and thus have the potential to help in preventing or treating of certain types of cancer, cardiovascular and neurodegenerative diseases, diabetes, and obesity (Khurana et al. 2013; Landete 2012; Pandey and Rizvi 2009). It is assumed that the described positive effects strongly depend on the bioavailability and the consumed amount of polyphenols (Saura-Calixto et al. 2007). In this context, access to individual polyphenolic compounds is of great interest for studying the presumed health-promoting effects in more detail. Unfortunately, only low amounts of individual polyphenols accumulate in plants and not all of these compounds are produced at all times as their biosynthesis often requires environmental triggers. Polyphenol compositions in plants strongly vary among different plant species and plant tissues, and are also subjected to seasonal and geographical variation. Thus, polyphenol extraction from the native plant is in most cases economically not feasible. Only in rare cases plant-extracted polyphenols made it to commercialization as dietary supplements, e.g., in case of the stilbene resveratrol or the flavonol quercetin. In most cases, chemical polyphenol synthesis is also not profitable as it involves complex reaction cascades, utilization or accumulation of toxic chemicals, and laborious purification (Quideau et al. 2011). Alternatively, functional introduction of plant-derived

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pathways in genetically engineered microbial host strains represents a promising strategy towards a more environmentally friendly polyphenol production from renewable and inexpensive resources.

In plants, the two major classes of polyphenols, stilbenoids and flavonoids, are derived from the ubiquitous aromatic amino acids L-phenylalanine (Phe) or L-tyrosine (Tyr). Both amino acids are initially converted to phenylpropanoids by non-oxidative deamination. In this reaction, phenylalanine ammonia lyases (PAL) and tyrosine ammonia lyases (TAL) convert Phe and Tyr to the phenylpropanoid cinnamic acid and *p*-coumaric acid, respectively (MacDonald and D'Cunha 2007; Rosler et al. 1997) (Fig. 1). Both phenylpropanoids can be further modified by hydroxylation- or *O*-methylation reactions, which gives rise to additional phenylpropanoids such as caffeic acid, ferulic acid, hydroxyferulic acid, and sinapic acid (Hahlbrock and Scheel 1989) (Fig. 1). For the synthesis of stilbenoids and flavonoids, phenylpropanoids as direct precursor molecules are first converted to their respective coenzyme A (CoA) thioesters. These ATP-dependent CoA ligation reactions are catalyzed by 4-coumarate: CoA ligases (4CL). Subsequently, two different type III polyketide synthases (stilbene synthases, STS or chalcone synthases, CHS) consume the phenylpropanoid CoA thioesters as starter units and catalyze three malonyl-CoA-dependent chain elongation steps yielding identical tetraketide intermediates, which are subsequently converted either into a stilbene (STS-catalyzed reaction) or into a chalcone (CHS-catalyzed reaction) (Tropf et al. 1994) (Fig. 1). Chalcone isomerases catalyze the subsequent isomerization of chalcones to (2*S*)-flavanones. The (2*S*)-flavanone naringenin is the first compound in the flavonoid pathway constituting the "flavonoid core", and represents the most important precursor molecule for almost all flavonoids. Naringenin can be further converted to dihydroflavonols, flavonols, isoflavones, and anthocyanidins (all belonging to the class of flavonoids) by

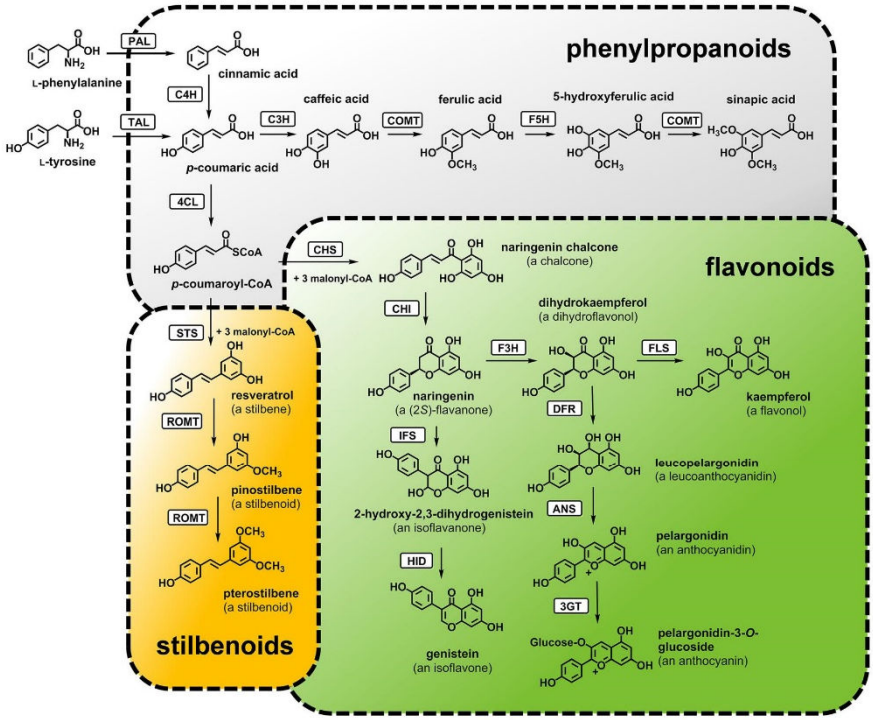

Fig. 1 Pathways for phenylpropanoid, stilbenoid, and flavonoid synthesis starting from aromatic amino acids. For the sake of simplicity, only the stilbenoids and flavonoids derived from *p*-coumaric acid are depicted. PAL phenylalanine ammonia lyase, C4H cinnamate 4-hydroxylase, TAL tyrosine ammonia lyase, C3H coumarate 3-hydroxylase, COMT caffeate *O*-methyltransferase, F5H ferulate 5-hydroxylase, 4CL 4-coumarate: CoA ligase, STS stilbene synthase, ROMT resveratrol-di-*O*-methyltransferase, CHS chalcone synthase, CHI chalcone isomerase, IFS 2-hydroxyisoflavanone synthase, HID 2-hydroxyisoflavanone dehydratase, F3H flavanone 3-hydroxylase, FLS flavonol synthase, DFR dihydroflavonol reductase, ANS anthocyanidin synthase, 3GT anthocyanidin 3-glycosyltransferase

2-oxoglutarate dioxygenases and reductases (Fig. 1). Flavonoids as well as stilbenes can be decorated with glycosyl, methyl, acetyl or other acyl groups forming a large set of different stilbenoids and flavonoids with different chemical properties (Ibrahim et al. 1998; Vogt and Jones 2000). Polyphenol decoration is typically associated with an increase in water solubility, bioavailability, or molecule stability (Xiao and Högger 2015).

Plants and microorganisms share a similar primary metabolism, which means that the microbial metabolism can in principle also provide aromatic amino acids and malonyl-CoA as polyphenol precursor molecules. This also applies for relevant cosubstrates used for decoration, e.g., the methyl donor *S*-adenosyl methionine (SAM) and the glycosyl donor UDP-glucose. Hence, microorganisms represent an attractive alternative production platform for polyphenols when the respective plant pathways consuming these precursor molecules can be functionally introduced.

In this review article, we summarize key advancements in engineering microorganisms towards polyphenol production of the last 15 years. During these years, researchers focused on the functional introduction of plant-derived enzymes into the microbial hosts and on the optimal connection of the respective microbial carbon metabolism to the heterologous polyphenol pathways. In some organisms also competing pathways, consuming polyphenol precursor metabolites had to be identified and abolished.

Functional introduction of plant-derived pathways enables polyphenol production in microorganisms

The first plant-derived polyphenols produced in a microorganism were the (2*S*)-flavanones naringenin and pinocembrin, which are derived from the phenylpropanoids *p*-coumaric acid and cinnamic acid, respectively. In one of the first studies, expression of heterologous genes coding for TAL from the yeast *Rhodotorula rubra*, 4CL from *Streptomyces coelicolor* and CHS from *Glycyrrhiza echinata* in *Escherichia coli* allowed for the accumulation of 0.5 mg/L naringenin and 0.8 mg/L pinocembrin from supplemented Tyr and Phe, respectively (Hwang et al. 2003). The product titer could be considerably increased in 2004, when genes coding for a TAL from *Rhodobacter sphaeroides* and for 4CL and CHS from *Arabidopsis thaliana* were expressed in *E. coli*. The obtained strain produced 21 mg/L naringenin starting from Tyr (Watts et al. 2004). The same group also reported production of 105 mg/L resveratrol from *p*-coumaric acid in an *E. coli* strain expressing an STS-encoding gene from peanut (*Arachis hypogaea*) (Watts et al. 2006). In 2003, it could be shown that *Saccharomyces cerevisiae* can also serve as host organism for polyphenol production. By expressing a *4cl* gene from *Populus sp*. in combination with an *sts* gene from grape (*Vitis vinifera*) 0.001 mg/L resveratrol could be produced (Becker et al. 2003). Starting from 2005, other compound classes of the large flavonoid family were tapped by demonstrating production of flavones and flavonols in *E. coli* and *S. cerevisiae* for the first time (Leonard et al. 2005; Leonard et al. 2006a; Leonard et al. 2006b).

Since 2009, other microbial host organisms such as *Corynebacterium glutamicum*, *Lactococcus lactis*, and *Streptomyces venezuelae* were successfully introduced as alternative hosts for the microbial production of polyphenols (Donnez et al. 2009; Kallscheuer et al. 2016c; Park et al. 2009). In case of *C. glutamicum*, the natural competence for degrading phenylpropanoid precursor molecules needed to be eliminated before the organism could be successfully employed for polyphenol synthesis (Kallscheuer et al. 2016a; Kallscheuer et al. 2016c).

Increasing the supply of malonyl-CoA for improved polyphenol production

In these first studies, it became obvious that supplemented aromatic amino acids or phenylpropanoids were never completely converted to the corresponding polyphenols. This finding was attributed to the low intracellular availability of malonyl-CoA, which was identified as major bottleneck during polyphenol production in engineered microorganisms (Miyahisa et al. 2005). Malonyl-CoA, produced from acetyl-CoA by an acetyl-CoA carboxylase (ACC), is for the most part endogenously consumed during chain elongation reactions in fatty acid biosynthesis. Hence, the intracellular level of malonyl-CoA is strictly regulated at the level of ACC activity to prevent any fatty acid overproduction (Brownsey et al. 2006; Tehlivets et al. 2007). Metabolic engineering of polyphenol-producing microorganisms aimed at enhancing the malonyl-CoA supply by increasing its biosynthesis and by decreasing its conversion during fatty acid biosynthesis.

A considerable increase in the obtained polyphenol titers could be observed in the presence of fatty acid biosynthesis inhibitors. In most studies, the natural fatty acid synthase-inhibiting antibiotic cerulenin was supplemented, which increased the production of polyphenols in engineered *E. coli* 3- to 20-fold (Leonard et al. 2008; Santos et al. 2011; van Summeren-Wesenhagen and Marienhagen 2015). A constructed *E. coli* strain expressing heterologous genes coding for a 4CL from *A. thaliana* and for an STS from *V. vinifera* produced 2.3 g/L of the stilbene resveratrol by a two-step biotransformation from *p*-coumaric acid in presence of cerulenin (Lim et al. 2011). This is the highest reported resveratrol titer obtained from the precursor *p*-coumaric acid in a microorganism to date. In *C. glutamicum*, the production of resveratrol increased 13-fold (final titer 158 mg/L) in presence of cerulenin (Kallscheuer et al. 2016c). Cerulenin is very expensive (20 € per mg), and thus, cannot be used in large-scale fermentations. Alternatively, fatty acid synthesis was down-regulated by posttranscriptional gene

silencing using antisense RNA (asRNA). Expression of an asRNA targeting the mRNA of the malonyl-CoA-acyl carrier protein (ACP) transacylase gene *fadD* resulted in a 4.5-fold increase of the intracellular malonyl-CoA concentration in *E. coli* (Yang et al. 2015). This in turn allowed for a 1.5-fold increase of the naringenin titer (91 mg/L) and a 1.7-fold increase of the resveratrol titer (268 mg/L). In a similar study, an increase of the naringenin titer from 91 to 391 mg/L (more than fourfold increase) was observed when the expression of the genes *fabH* and *fapB* was downregulated by posttranscriptional gene silencing (Wu et al. 2014a). FabH and FabB are 3-oxoacyl-ACP synthases, which catalyze the initial malonyl-CoA-dependent elongation reaction of the iterative fatty acid synthesis (Tsay et al. 1992). More recently, expression of genes involved in fatty acid biosynthesis was down-regulated by using CRISPRi, which increased the obtained polyphenol titer of naringenin sevenfold (final titer 422 mg/L) and of the stilbene pinosylvin twofold (final titer 47 mg/L) (Cress et al. 2015; Liang et al. 2016; Wu et al. 2015).

Improved supply of malonyl-CoA typically relies on increasing the ACC activity for enhancing the conversion of acetyl-CoA to malonyl-CoA. Alternatively, a malonate assimilation pathway from *Rhizobium trifolii* was used, in which malonate is initially converted to malonyl-CoA by CoA-ligation (An and Kim 1998). Two of the involved genes coding for a malonate carrier protein (*matC*) and a malonyl-CoA synthetase (*matB*) were exploited for increased polyphenol production from supplemented malonate (Fig. 2). By following this strategy in *E. coli*, the production of the (2*S*)-flavanone pinocembrin was increased up to 15-fold (final titer 480 mg/L) (Leonard et al. 2008; Wu et al. 2013). This approach requires the supplementation of malonate as precursor

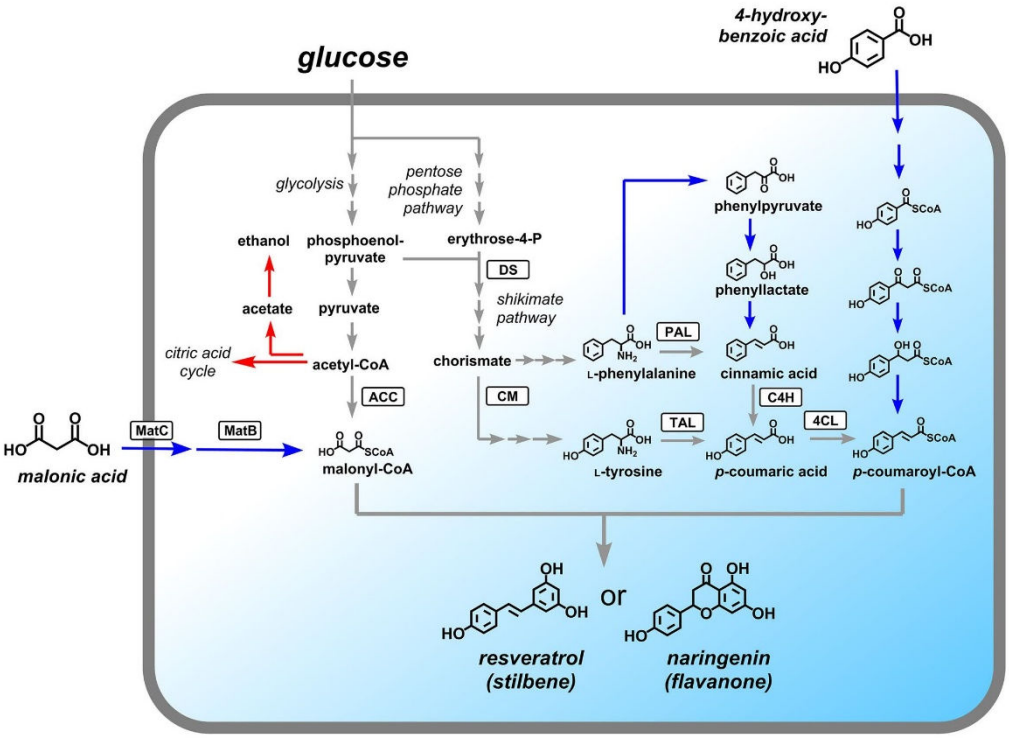

Fig. 2 Overview of engineered natural and non-natural pathways supplying *p*-coumaroyl-CoA and malonyl-CoA as polyphenol precursor molecules. The relevant natural pathways for the synthesis of aromatic amino acids (shikimate pathway), phenylpropanoids (ammonia-lyase reaction), and malonyl-CoA (carboxylation of acetyl-CoA) are indicated by gray arrows. Red arrows indicate competing reactions consuming acetyl-CoA, which are either abolished or downregulated in engineered microbial producer strains. The orthogonal pathway for malonyl-CoA (malonic acid assimilation pathway from *Rhizobium trifolii)* and the functional non-natural pathways leading to cinnamic acid or *p*-coumaroyl-CoA are indicated by blue arrows. ACC acetyl-CoA carboxylase, MatC malonate carrier protein, MatB malonyl-CoA synthetase, DS 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, CM chorismate mutase, PAL phenylalanine ammonia lyase, TAL tyrosine ammonia lyase, C4H cinnamate 4-hydroxylase, 4CL 4-coumarate: CoA ligase

and was also functionally implemented in other organisms such as *S. venezuelae* (Park et al. 2011).

Alternatively, for an increased ACC activity, four genes encoding a heterotetrameric ACC along with a gene for a necessary biotin ligase from *Photorhabdus luminescens* were heterologously expressed in *E. coli* (Leonard et al. 2007). This strategy allowed for a sevenfold increase of the microbial pinocembrin production from supplemented cinnamic acid with a maximal product titer of 196 mg/L (Leonard et al. 2007). Alternatively, the heterodimeric enzyme AccBC-AccD1 of *C. glutamicum* was employed as a functional ACC in *E. coli* as it only requires expression of two genes (Gande et al. 2007; Miyahisa et al. 2005). This strategy boosted the intracellular malonyl-CoA concentration in *E. coli* 15-fold and allowed for the accumulation of 1.3 g/L phloroglucinol (Zha et al. 2009). In contrast, the monomeric ACC of *S. cerevisiae* is a multi-domain protein encoded by the gene *acc1*. Overexpression of *acc1* improved the resveratrol production in *S. cerevisiae* twofold to 6 mg/L (Shin et al. 2012). ACC1 activity was found to be repressed by protein kinase-dependent phosphorylation of two serine side chains. Substitution of serine for alanine at both positions abolished the posttranslational regulation and led to increased levels of malonyl-CoA (Shi et al. 2014).

The central carbon metabolism of polyphenol-producing *E. coli* strains was additionally engineered towards increased acetyl-CoA supply. The elimination of pathways for acetate and ethanol formation, interruption of the citric acid cycle, and an increased flux from glucose to pyruvate proved to be suitable strategies for increasing acetyl-CoA supply (Fowler et al. 2009; Xu et al. 2011; Zha et al. 2009) (Fig. 2). Combination of genetic modifications ensuring increased ACC activity and higher intracellular acetyl-CoA availability yielded an *E. coli* strain capable of producing 470 mg/L naringenin from supplemented *p*-coumaric acid (Xu et al. 2011).

Engineering of the microbial aromatic amino acid metabolism enables phenylpropanoid and polyphenol synthesis from glucose

In initial studies, engineered microorganisms typically produced the desired plant polyphenols from supplemented phenylpropanoids or amino acids as precursor molecules (Lussier et al. 2012). However, microbial production from cheap substrates such as glucose or ethanol would be more economical. Metabolic engineering into this direction included two major goals: (i) increasing the carbon flux into the shikimate pathway for the biosynthesis of the aromatic amino acids Phe or Tyr and (ii) the functional introduction of ammonia lyases for the efficient conversion of aromatic amino acids to the respective phenylpropanoids (Fig. 2). Similar to other amino acid biosynthetic pathways, the shikimate pathway is mainly regulated by feedback inhibition of allosterically controlled key enzymes (Herrmann and Weaver 1999; Ikeda 2006). In most microorganisms, the aromatic amino acids inhibit the activity of the 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (DS), which catalyzes the initial committed step of the pathway (Fig. 2). In *E. coli*, an increased flux into the shikimate pathway was achieved by employing feedback-resistant DS variants (Ger et al. 1994; Jossek et al. 2001; Ray et al. 1988). Introduction of a feedback-resistant DS in combination with a feedback-resistant chorismate mutase/prephenate dehydrogenase (catalyzing the reaction at the metabolic branch leading to Tyr) and a TAL enabled coupling of the endogenous primary metabolism to the heterologous polyphenol pathway. In *E. coli*, naringenin was produced from glucose with maximal titers of 80–100 mg/L (Santos et al. 2011; Wu et al. 2014b). By following the same strategy for elevated intracellular Phe availability, production of 40 mg/L pinocembrin from glucose could be established (Wu et al. 2013). The production of sakuranetin (7-*O*-methylnaringenin) and ponciretin (4′-*O*-methylnaringenin) as more complex flavonoids from glucose in *E. coli* required heterologous expression of altogether eight genes (Kim et al. 2013). In this study, synthesis of 40 mg/L sakuranetin and 43 mg/L ponciretin could be observed. In *C. glutamicum*, deregulation of the shikimate pathway and introduction of a heterologous TAL from *Flavobacterium johnsoniae* enabled the production of 60 mg/L resveratrol and 32 mg/L naringenin from glucose (Kallscheuer et al. 2016c). In *S. cerevisiae*, the same strategy as in bacteria could be successfully adopted for overproducing aromatic amino acids as polyphenol precursor molecules (Rodriguez et al. 2015). In addition to these genetic modifications, the Ehrlich pathway competing for aromatic amino acids was abolished. The resulting yeast strain proved to be a suitable platform for the production of phenylpropanoid-derived compounds from glucose as accumulation of 2 g/L *p*-coumaric acid could be observed. In a follow-up study, *S. cerevisiae* was further engineered for the production of resveratrol by introducing genes for 4CL and STS enzyme activities and by increasing the malonyl-CoA supply (Li et al. 2016). The best-performing strain accumulated 800 mg/L resveratrol, which is the highest reported polyphenol titer obtained from glucose in an engineered microorganism to date.

Especially in bacterial production strains, low activity of the heterologously introduced PALs or TALs was found to be another bottleneck limiting overall polyphenol production from glucose (Eudes et al. 2013; Kallscheuer et al. 2016c; Lin and Yan 2012). This is most probably related to the enzyme-catalyzed reaction mechanism, which includes elimination of the non-acidic proton at the C3 carbon and was proposed to be associated with a dearomatization of the aromatic ring of the substrate (MacDonald and D'Cunha 2007). In a comparative in vivo study, ammonia lyases from various donor organisms were evaluated in the three microbial platform organisms *E. coli*, *S. cerevisiae*, and *L. lactis*, and several

enzymes exhibiting high activity and substrate specificity could be identified (Jendresen et al. 2015). In plants, no ammonia lyase-independent route to phenylpropanoids is known. More recently, bio-inspired, synthetic pathways leading to these polyphenol precursor molecules were designed in silico and successfully implemented into microbial production strains (Fig. 2). In *C. glutamicum*, the use of a CoA-dependent, β-oxidative phenylpropanoid catabolic pathway from *Azoarcus sp.* EbN1 was exploited in the non-natural anabolic direction for conversion of supplemented 4-hydroxybenzoic acid to *p*-coumaroyl-CoA, which was subsequently used for the microbial production of resveratrol (Kallscheuer et al. 2016b). In *E. coli*, a PAL-independent α-reductive pathway starting from phenylpyruvate yielded cinnamic acid via the pathway intermediate phenyllactate (Masuo et al. 2016) (Fig. 2).

Decoration of polyphenols in microbial production strains

Plants synthesize a large diversity of several thousand polyphenols by decoration of only a few stilbenoid and flavonoid backbone molecules. Decoration of polyphenols results from hydroxylation and subsequent *O*-methylation, *O*-acylation, or glycosylation reactions, which give rise to polyphenols with altered chemical properties influencing, e.g., water solubility and overall molecule stability (Tsao 2010). Initially, metabolic engineering of microorganisms for polyphenol production focused on the synthesis of stilbenoid and flavonoid backbone molecules, but scientists also started to tap the large natural polyphenol diversity by implementing downstream reactions for polyphenol decoration in the microbial production strains. Depending on the pathway and the hydroxy group to be decorated, required reactions can either be included at the earlier stage of phenylpropanoid precursor synthesis or at a later stage when the polyphenol core structure is available.

At the stage of phenylpropanoids, engineering efforts focused on the synthesis of *O*-methylated compounds such as ferulic acid and sinapic acid (Fig. 1) as these phenylpropanoids also serve as precursors for other compounds related to polyphenols (e.g., lignans, coumarins, and curcumins) (Marienhagen and Bott 2013). For this purpose, the phenylpropanoid *p*-coumaric acid was hydroxylated to caffeic acid in *E. coli* (Furuya and Kino 2014; Huang et al. 2013). Depending on the amount of supplemented *p*-coumaric acid, up to 10.2 g/L caffeic acid could be produced, whereas the highest product titer obtained from glucose was 0.8 g/L. With an additional caffeate *O*-methyltransferase, 0.2 g/L ferulic acid could be produced from glucose (Kang et al. 2012), while sinapic acid synthesis could not be achieved in microorganisms yet.

Hydroxylation of polyphenol core structures in plants is mediated by membrane-bound cytochrome P450-dependent monooxygenases, which are difficult to functionally introduce into microorganisms (Rodrigues et al. 2015). In *E. coli*, translational fusion of a flavonoid 3′,5′-hydroxylase with a P450 reductase enabled production of flavonols with different hydroxylation patterns (Leonard et al. 2006b). The same group also published the functional introduction of a P450-dependent isoflavone synthase in *E. coli* and *S. cerevisiae* and demonstrated the production of the isoflavone genistein in both organisms (Leonard and Koffas 2007). Furthermore, the pathway for the flavonol fisetin, in which the intermediate resokaempferol is hydroxylated to fisetin in the last step, was reconstructed in *E. coli* (Stahlhut et al. 2015). In this study, the translational fusion of a truncated version of a flavonoid 3′-monooxygenase from *A. thaliana* with a truncated version of a cytochrome P450 reductase from *Catharanthus roseus* allowed for a fisetin titer of 1.2 mg/L. *C. glutamicum* was found to be a suitable microbial host organism for the production of flavonols. The functional introduction of two 2-oxoglutarate-dependent dioxygenases (flavanone 3-hydroxylase and flavonol synthase) in a (2*S*)-flavanone-producing *C. glutamicum* strain was key for developing a strain capable of accumulating the highest reported flavonol concentrations of 23 mg/L kaempferol and 10 mg/L quercetin from supplemented *p*-coumaric acid and caffeic acid, respectively, in the culture medium (Kallscheuer et al. 2017).

O-methylation of the stilbene resveratrol was achieved in *E. coli* and *C. glutamicum* by functional introduction of different *O*-methyltransferases. This allowed for the production of the mono-, di-, and tri-*O*-methylated resveratrol derivatives with maximal titers of 50 mg/L (Heo et al. 2017; Jeong et al. 2014; Kallscheuer et al. 2017; Kang et al. 2014; Wang et al. 2015b). In a similar manner, the introduction of glycosyltransferases from various donor organisms enabled production of glycosylated resveratrol derivatives comprising the 4′-*O*-glucoside and the 3-*O*-glucoside in *E. coli* (Choi et al. 2014; Ozaki et al. 2012). Anthocyanins, the most abundant flavonoids in fruits and vegetables, are glycosylated as this decoration improves the molecule stability compared to the very unstable anthocyanin aglycones referred to as anthocyanidins (Vogt and Jones 2000). For the production of anthocyanins in *E. coli*, genes coding for a UDP-glucose: flavonoid 3-*O*-glucosyltransferase from *Petunia hybrida* and *A. thaliana* were expressed and cultivations were performed under acidic conditions as anthocyanins show higher stability at a lower pH (Yan et al. 2008). Under optimized conditions, anthocyanin titers of 70–80 mg/L could be achieved. Very recently, more complex anthocyanins such as peonidin 3-*O*-glucoside were produced (Cress et al. 2017), while production of 10 mg/L pelargonidin-3-*O*-glucoside from glucose was demonstrated

using a four-strain *E. coli* polyculture collectively expressing 15 heterologous genes (Jones et al. 2017).

E. coli was used as a glycosylation platform supplying different UDP- or TDP-activated sugars. By using this strategy, several glycosylated flavonol derivatives were produced when the respective aglycone was supplemented (De Bruyn et al. 2015; Pandey et al. 2013; Simkhada et al. 2010; Yang et al. 2014). For the glycosylation of various flavonoids also bacterial glycosyltransferases from *Bacillus cereus* or *Xanthomonas campestris* were successfully employed, e.g., for the synthesis of kaempferol 7-*O*-glucoside, quercetin 3-*O*-glucoside, and luteolin-3′-*O*-glucoside (Hyung Ko et al. 2006; Kim et al. 2007). In a recent study, the glycosyl donor metabolite dTDP-glucose was modified by an additional dehydratase and an aminotransferase, which enabled synthesis of the non-natural compound fisetin 3-*O*-4-amino-4,6-dideoxy-galactoside (Pandey et al. 2016). This recent study demonstrates that manipulation of pathways for the respective sugar donors can be used to carry out tailor-made decorations, which eventually lead to synthetic polyphenol glycosides. In parallel, supplementation of different precursor metabolites enabled synthesis of nearly 100 different polyphenol aglycones and half of these represent non-natural compounds (Chemler et al. 2007; Katsuyama et al. 2007). Taken together, the great natural diversity of polyphenols can even be surpassed by combining non-natural polyphenols with modified sugar residues. When considering that polyphenols can carry several decorations, one can imagine that a very large variety of compounds with different properties can be made accessible by this approach.

Table 1 Production of phenylpropanoids, stilbenoids and flavonoids in engineered microorganisms

Product	Product titer		Precursor	Precursor titer		Organism	Special conditions	Reference
	[mg/L]	[mM]		[mg/L]	[mM]			
Phenylpropanoids								
p-Coumaric acid	1930	11.8	None, from glucose	–	–	*S. cerevisiae*		Rodriguez et al. 2015
Caffeic acid	767	4.2	None, from glucose	–	–	*E. coli*		Huang et al. 2013
Caffeic acid	10,200	56.6	*p*-Coumaric acid	16,400	100	*E. coli*		Furuya and Kino 2014
Ferulic acid	196	1.0	None, from glucose	–	–	*E. coli*		Kang et al. 2012
Stilbenoids								
Pinosylvin	91	0.43	Phenylalanine	496	3	*E. coli*	Addition of cerulenin	van Summeren-Wesenhagen and Marienhagen 2015
Pinosylvin	121	0.57	Cinnamic acid	741	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2016c
Resveratrol	2340	10.3	*p*-Coumaric acid	2462	15	*E. coli*	Addition of cerulenin	Lim et al. 2011
Resveratrol	812	3.6	None, from glucose	–	–	*S. cerevisiae*		Li et al. 2016
Piceatannol	65	0.27	Resveratrol	100	0.44	*E. coli*		Wang et al. 2015a
Piceatannol	56	0.23	Caffeic acid	900	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2016c
Pinostilbene	34	0.14	Resveratrol	228	1	*E. coli*		Jeong et al. 2014
Pterostilbene	34	0.13	None, from glucose	–	–	*E. coli*		Heo et al. 2017
Pterostilbene	170	0.66	Resveratrol	228	1	*E. coli*		Wang et al. 2015b
(2S)-Flavanones								
Pinocembrin	710	2.8	Cinnamic acid	296	2	*E. coli*	Addition of cerulenin	Leonard et al. 2008
Pinocembrin	432	1.7	None, from glucose	–	–	*E. coli*	Malonate assimilation	Wu et al. 2016
Naringenin	474	1.7	*p*-Coumaric acid	426	2.6	*E. coli*		Xu et al. 2011
Naringenin	391	1.4	L-Tyrosine	543	3	*E. coli*	Malonate assimilation	Wu et al. 2014a
Naringenin	84	0.31	None, from glucose	–	–	*E. coli*	Addition of cerulenin	Santos et al. 2011
Eriodictyol	107	0.37	L-Tyrosine	181	1	*E. coli*		Zhu et al. 2014
Flavones								
Chrysin	9	0.04	L-Phenylalanine	543	3	*E. coli*		Miyahisa et al. 2006
Chrysin	31	0.12	Cinnamic acid	178	1.2	*S. venezuelae*	Malonate assimilation	Park et al. 2011
Apigenin	30	0.11	*p*-Coumaric acid	49	0.3	*E. coli*		Lee et al. 2015
Apigenin	15	0.06	*p*-Coumaric acid	197	1.2	*S. venezuelae*	Malonate assimilation	Park et al. 2011
Flavonols								
Kaempferol	66	0.23	*p*-Coumaric acid	164	1	*S. cerevisiae*	Fed-batch cultivation	Duan et al. 2017
Kaempferol	23	0.08	*p*-Coumaric acid	820	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2017
Quercetin	10	0.03	Caffeic acid	900	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2017
Isoflavones								
Genistein	8	0.03	Naringenin	136	0.5	*S. cerevisiae*		Trantas et al. 2009
Anthocyanins								
Pelargonidin-3-*O*-glucoside	79	0.18	(+)-Afzelechin	206	0.75	*E. coli*		Yan et al. 2008
Cyanidin-3-*O*-glucoside	350	0.78	(+)-Catechin	1016	3.5	*E. coli*		Lim et al. 2015

Conclusion and outlook

Major breakthroughs in this field allowed for the construction of microbial cell factories for a broad range of different plant-derived polyphenols in the last 15 years. However, whereas flavonoid and stilbene core structures can now be produced at (almost) gram-scale from cheap glucose (Table 1), microbial synthesis of more complex polyphenol structures remains challenging. In such cases, accumulation of only low product concentrations usually impedes any commercial production. At least a tenfold increase in the current polyphenol titers would be necessary to render microbial production of these compounds economically feasible. Major limitations are (still) insufficient supply of precursor molecules by the microbial metabolism and low activity of plant-derived enzymes in heterologous hosts. However, recent technological innovations such as the development of biosensor-driven directed evolution approaches could enable rapid engineering of microbial host strains for providing more precursor molecules to increase polyphenol synthesis (Schallmey et al. 2014; Siedler et al. 2017). New molecular techniques for the rapid assembly of biosynthetic pathways and balancing of (heterologous) gene expression in the metabolic context of the microbial host could improve rerouting of carbon fluxes to and through polyphenol-providing pathways of interest (van Summeren-Wesenhagen et al. 2015). Similarly, approaches and concepts from synthetic biology allowing for dynamic pathway regulation and metabolic control could be also employed to harmonize endogenous and heterologous pathways for maximizing product titers (Xu et al. 2014). First examples of new pathways for polyphenol synthesis, not found in nature, have been presented and more functional synthetic routes from the scientist's drawing board are expected to become available in the future (Kallscheuer et al. 2016b). Furthermore, protein engineering strategies might help to improve the performance of plant enzymes in the respective microbial host and chemical diversification of precursor molecules or the final polyphenol products might give rise to compounds with new properties. No less important than the genetic design of the microbial cell factories is the aspect of process engineering as optimization and balancing of microbial growth and product formation are decisive for success. In addition, in situ product removal for preventing undesired oxidation of the target compounds and for minimizing potentially toxic effects of the polyphenols on the microbial host will be of increasing importance as the obtained product titers also increase (Braga et al. 2017).

Future developments require joint efforts of multiple disciplines such as biology, biochemistry, chemistry, process engineering, and other disciplines (Dudnik et al. 2017). For this reason, collaborative projects in which scientists with different backgrounds and ideas work on the goal of an economically feasible microbial polyphenol production are of great importance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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2.2 Microbial production of natural and non-natural monolignols with *Escherichia coli*

ChemBioChem 10.1002/cbic.201800673

Microbial production of natural and non-natural monolignols with *Escherichia coli*

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Abstract

Phenylpropanoids and phenylpropanoid-derived plant polyphenols find numerous applications in food and pharmaceutical industries. In recent years, several microbial platform organisms were engineered towards producing such compounds. However, for the most part, microbial (poly)phenol production is inspired by nature, and thus predominantly naturally occurring compounds have been produced to this date.

Here, we took advantage of the promiscuity of enzymes involved in phenylpropanoid synthesis and exploited the versatility of an engineered *E. coli* strain harboring a synthetic monolignol pathway to convert supplemented natural and non-natural phenylpropenoic acids to their corresponding monolignols. Performed biotransformations showed that this strain is able to catalyze the stepwise reduction of chemically interesting non-natural phenylpropenoic acids such as 3,4,5-trimethoxycinnamic acid, 5-bromoferulic acid, 2-nitroferulic acid, and a 'bicyclic' *p*-coumaric acid derivative in addition to six naturally occurring phenylpropenoic acids.

Keywords

biocatalysis, *p*-coumaric acid, monolignols, natural products, phenylpropanoids

ChemBioChem 10.1002/cbic.201800673

Introduction

Many plant polyphenols such as flavonoids, stilbenes or lignans are important compounds for the food and pharmaceutical industries.[1] Here they find an application, e.g., as flavors, colorants, therapeutic agents or antibiotics. General precursor molecules of these valuable compounds are phenylpropanoids, which in turn are derived from the aromatic amino acids L-phenylalanine or L-tyrosine (**1**). Phenylpropanoid synthesis starts with the non-oxidative deamination of the aromatic amino acid yielding the typical phenylpropanoid core structure: a phenyl group attached to a propene tail (Figure 1).[1–3] This decisive reaction is catalyzed by ammonia lyases, either phenylalanine ammonia lyases (PAL) or tyrosine ammonia lyases (TAL).

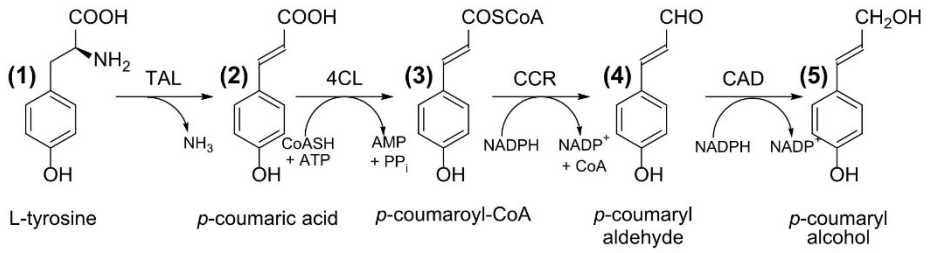

Figure 1. Biosynthetic pathway for *p*-coumaryl alcohol synthesis from L-tyrosine. TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase.

In case of L-tyrosine, the resulting phenylpropenoic acid *p*-coumaric acid (**2**) is subsequently activated by 4-coumarate-CoA ligases (4CL) yielding 4-coumaroyl-CoA. This CoA-activated compound **3** can subsequently serve as precursor molecule for the synthesis of flavonoids and stilbenes. Alternatively, *p*-coumaroyl-CoA can be stepwise reduced to the respective alcohol **5**, which is also referred to as monolignol. The required two reduction steps are catalyzed by cinnamoyl-CoA reductases (CCR) and

ChemBioChem 10.1002/cbic.201800673

cinnamyl alcohol dehydrogenases (CAD), respectively. In plants the resulting monolignols represent key building blocks for the synthesis of lignin, but are also necessary for the synthesis of the pharmaceutically interesting group of lignans.[4,5]

In principle, phenylpropanoids and phenylpropanoid-derived polyphenols can be isolated from plants as their natural producers, but polyphenol concentrations in the plant usually account for less than one percent of the plant dry weight only.[6] Furthermore, plant extraction is also limited by slow plant growth as well as environmental and regional factors affecting overall product yields.[7,8] Total chemical synthesis represents an interesting alternative, but depending on the complexity of the target compound the synthesis route comprises a number of individual steps with intermediate purifications.[9–11] Microbial phenylpropanoid production offers a promising alternative to the uneconomic isolation from plant material as modern molecular tools allow for the functional implementation of plant biosynthetic pathways into the microbial metabolism.[1] Following this strategy, many microbial strains for plant phenol synthesis were developed in recent years, especially for the production phenylpropanoid-derived flavonoids and stilbenes.[12,13]

In this context, an *Escherichia coli* strain has been engineered to accumulate up to 52 mg/L *p*-coumaryl alcohol (**5**) without supplementation of any precursor molecules.[14] The strain harbors a full synthetic phenylpropanoid pathway, which is plasmid-encoded by a tetracistronic operon. Interestingly, all four enzymes participating in monolignol biosynthesis have been previously described to be promiscuous with regard to their substrate specificities.[15] This finding could enable biosynthesis of other natural, and possibly also non-natural monolignols with interesting applications from supplemented precursor molecules.[15,16] However, practicability of this concept has been only demonstrated for the microbial production

ChemBioChem 10.1002/cbic.201800673

of cinnamyl alcohol (**6**), caffeoyl alcohol (**7**) and coniferyl alcohol (**8**) from supplemented natural cinnamic acid derivatives.[17–19] In addition, individual enzymes of the monolignol pathway were successfully used for the microbial synthesis of different non-natural flavanones and stilbenes from various precursors.[20–25] Here, more detailed studies exploring the catalytic promiscuity of the enzymes of the monolignol pathway will not only help to gain a deeper understanding of the enzymes involved, but might also provide access to new compounds with interesting chemical or pharmaceutical properties.[26–30]

In this study, we set out to explore the catalytic versatility of a synthetic monolignol pathway in *E. coli* by supplementing naturally and non-natural occurring cinnamic acid derivatives.

Results and Discussion

Microbial synthesis of naturally occurring monolignols with *E. coli*

Recently, *E. coli* BL21-Gold (DE3) *lacI*^{Q1} pALXtreme-*tal-4cl-ccr-cad* was designed and constructed, which can synthesize the monolignol *p*-coumaryl alcohol (**5**).[14] This strain harbors a synthetic monolignol pathway composed of a tyrosine ammonia lyase from *Rhodobacter sphaeroides* (TAL_{Rs}, GenBank: ABA81174.1), a 4-coumarate: CoA ligase from *Petroselinum crispum* ($4CL_{Pc}$, GenBank: X13324.1), a cinnamoyl-CoA reductase from *Zea mays* (CCR_{Zm}, GenBank: Y15069.1) and a cinnamyl-alcohol dehydrogenase from *Z. mays*. All four genes, organized as synthetic operon under control of the IPTG-inducible T7 promoter are plasmid-encoded. Initially, it was tested, if this monolignol pathway is also capable of reducing cinnamic acid (**9**), caffeic acid (**10**), ferulic acid

ChemBioChem 10.1002/cbic.201800673

(**11**), hydroxyferulic acid (**12**) and sinapic acid (**13**) as the most abundant naturally occurring cinnamic acid derivatives (Figure 2).

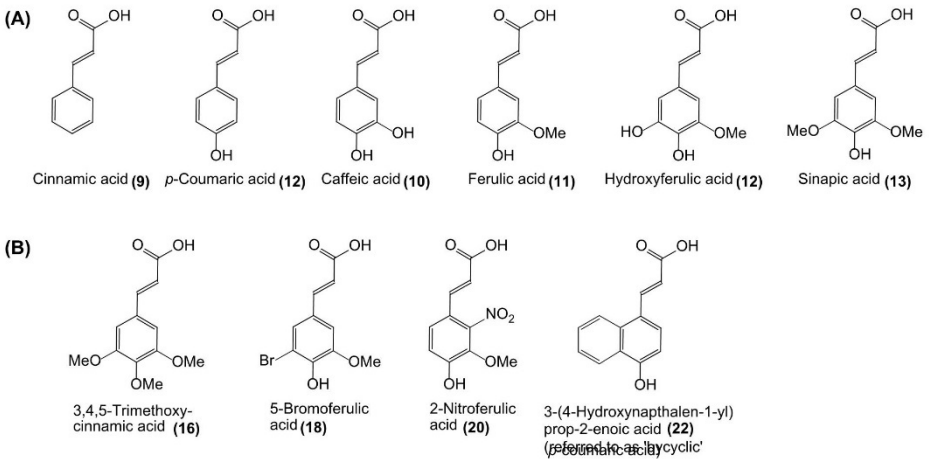

Figure 2. (A) Naturally occurring and **(B)** non-natural cinnamic acid derivatives used in this study.

For this purpose, all phenylpropenoic acids were individually supplemented to cultures of growing *E. coli* cells at a concentration of 2.5 mM right at the start of the cultivation. After 17 hours of cultivation, the concentrations of supplemented acid precursor molecules as well as their corresponding monolignols in the supernatant were determined by HPLC.

As a result of this systematic approach, it could be confirmed that the synthetic pathway, although comprised of enzymes originating from three different organisms, is indeed capable to reduce all supplemented natural cinnamic acid derivatives to their corresponding monolignols in *E. coli* (Table 1). In case of cinnamic acid **(9)** as chemically “most simple” precursor without any additional substituent on the aryl ring, a product titer of 195 mg/L (1.46 mM) cinnamyl alcohol **(6)** could be determined (Table 1). In the past, cinnamyl alcohol **(6)** was produced in *E. coli* with a different set of enzymes yielding 300 mg/L (2.24 mM) after 24 h.[17] However, biotransformations in

ChemBioChem 10.1002/cbic.201800673

this study were performed using TB media containing a glycerol/glucose mixture (1 g/L), which served as carbon and energy source as this turned out to be the most suitable medium for monolignol synthesis with *E. coli* in previous studies.[14]

Table 1. Monolignol titers obtained through biotransformations with *E. coli* BL21-Gold (DE3) *lacI*^{Q1} pALXtreme-*tal-4cl-ccr-cad* from supplemented phenylpropenoic acids. For production, *E. coli* cells were cultivated in 50 mL LB medium and 2.5 mM of the respective cinnamic acid derivatives were individually supplemented. All biotransformations were performed at 25 °C for 17 h. Data represents average values and standard deviations from three biological replicates.

	Monolignol concentration	
	[mg/L]	[mM]
Natural monolignols		
Cinnamyl alcohol	195 ± 62	1.46
p-Coumaryl alcohol	121 ± 5	0.81
Caffeoyl alcohol	5 ± 1	0.03
Coniferyl alcohol	327 ± 10	1.82
Hydroxyconiferyl alcohol	102 ± 30	0.52
Sinapyl alcohol	30 ± 3	0.14
Non-natural monolignols		
3,4,5-Trimethoxycinnamyl alcohol	4 ± 1	0.02
5-Bromoconiferyl alcohol	462 ± 40	1.78
2-Nitroconiferyl alcohol	74 ± 15	0.33
'Bicyclic' *p*-coumaryl alcohol	25 ± 44	0.13

In addition to cinnamyl alcohol (**6**), the already described capability of this strain to produce *p*-coumaryl alcohol (**5**) could be confirmed as a concentration 121 mg/L (0.81 mM) of this monolignol could be determined in culture supernatants under the cultivation conditions described. Caffeic acid (**10**), characterized by an additional *O*-

ChemBioChem 10.1002/cbic.201800673

methyl group on the aryl-ring in comparison to *p*-coumaric acid, was the least favored substrate for the synthetic pathway as only 5 mg/L (0.03 mM) caffeoyl alcohol (**7**) accumulated in the supernatant. In previous studies, the microbial production of caffeoyl alcohol (**7**) with *E. coli* was achieved by using immobilized cells.[18] The engineered strain equipped with a different set of enzymes produced up to 39 mg/L (0.24 mM) caffeoyl alcohol (**7**) in LB medium within eight hours. In another recent study, 534 mg/L (3.22 mM) caffeoyl alcohol (**7**) could be produced with an engineered *E. coli* strain, but in total 4 mM caffeic acid (**10**) were supplemented at several time points during 22 hours of cultivation using an optimized cultivation protocol and M9-medium with yeast extract supplementation.[19] Interestingly, ferulic acid (**11**) turned out to be the preferred natural substrate in this study since a product titer of 327 mg/L (1.82 mM) of the corresponding coniferyl alcohol (**8**) could be determined in our experiments. Extraction of coniferyl alcohol (**8**) from one liter culture supernatant yielded 280 mg (1.55 mmol) of the pure compound.

For the first time the microbial production of hydroxyconiferyl alcohol (**14**) and sinapyl alcohol (**15**) from supplemented hydroxyferulic acid (**12**) and sinapic acid (**13**), respectively, could be demonstrated *in vivo*. After 17 h of cultivation, monolignol concentrations of 102 mg/L (0.52 mM) and 30 mg/L (0.14 mM), respectively could be determined (Table 1).

Noteworthy, not converted phenylpropenoic acids were not degraded and could be detected in the supernatants of the *E. coli* cultures (data not shown).

Microbial synthesis of non-natural monolignols with *E. coli*

Hitherto, only the microbial synthesis of naturally occurring monolignols has been described. This is somewhat surprising, as access to non-natural monolignols would

ChemBioChem 10.1002/cbic.201800673

also enable the synthesis pharmaceutically interesting lignans with novel properties. With the aim to explore the catalytic flexibility of the established synthetic pathway for the synthesis of such compounds, we attempted the conversion of four structurally very different non-natural phenylpropenoic acids in order to probe the scope of the approach. In particular, 5-bromoferulic acid (**18**) and 2-nitroferulic acid (**20**) were chosen based on their potential for further diversification, e.g., through palladium-catalyzed cross-couplings or after reduction to the corresponding aniline derivative. Among these, 3,4,5-trimethoxycinnamic acid (**16**) and 5-bromoferulic acid (**18**) were commercially available, but the substrates 2-nitroferulic acid (**20**) and 3-(4-hydroxynaphthalen-1yl)prop-2enoic acid (**22**) needed to be synthesized (see Supporting Information). In addition, the corresponding monolignols of all four non-natural substrates tested were chemically synthesized to serve as reference compounds for qualitative and quantitative analyses (see Supporting Information).

First experiments with 3,4,5-trimethoxycinnamic acid (**16**), a compound closely related to sinapic acid (**13**) revealed that only a small fraction of 0.02 mM (4 mg/L) of this substrate could be efficiently reduced to 3,4,5-trimethoxycinnamyl alcohol (**17**) (Table 1). In contrast, 5-bromoferulic acid (**18**) was rapidly reduced by the synthetic monolignol pathway and a final product titer of 462 mg/L (1.78 mM) 5-bromoconiferyl alcohol (**19**) could be determined in the supernatant (Table 1). Interestingly, under the conditions tested, 5-bromoferulic acid (**18**) proved to be a much better substrate compared to any of the naturally occurring phenylpropenoic acids used in this study. The engineered *E. coli* strain also successfully reduced 2-nitroferulic acid (**20**) to 2-nitroconiferyl alcohol (**21**). After 17 hours of biotransformation 74 mg/L (0.33 mM) 2-nitroconiferyl alcohol (**21**) accumulated in the supernatant (Table 1). Motivated by these results, the conversion of 'bicyclic' *p*-coumaric acid (**22**) as sterically most challenging substrate was also attempted (Figure 2). This naphthalene derivative also

ChemBioChem 10.1002/cbic.201800673

proved to be a suitable substrate as 25 mg/L (0.13 mM) of the corresponding monolignol 'bicyclic' *p*-coumaryl alcohol **23** could be detected in culture supernatants (Figure 1). Noteworthy, qualitative NMR experiments revealed that the pathway intermediate 'bicyclic' *p*-coumaryl aldehyde accumulated in the supernatants of the *E. coli* cultures. This indicates that this aldehyde is not a favored substrate for the CAD, which catalyzes the last reduction step of the synthetic monolignol pathway.

Optimization of the microbial 5-bromoconiferyl alcohol production

Subsequently, the 5-bromoconiferyl alcohol (**19**) production with the engineered *E. coli* strain was further optimized. Until this point substrate concentrations of 2.5 mM were used in all biotransformations since natural cinnamic acid derivatives are known to have an inhibitory effect on microbial growth.[31,32] With the aim to balance microbial growth and product yield, biotransformations with different 5-bromoferulic acid (**18**) concentrations were performed in 48-well microtiter plates in a microbioreactor system.

Unfortunately, 5-bromoferulic acid (**18**) concentrations exceeding 4 mM led to substrate precipitation, which rendered determination of the culture backscatter over time impossible (data not shown). This in turn impeded the evaluation of the impact of elevated substrate concentrations on microbial growth. However, performed cultivation experiments with substrate concentrations ranging from 0 mM and 2.5 mM already revealed, that presence of 5-bromoferulic acid (**18**) has a growth-inhibiting effect similar to the naturally phenylpropenoic acids tested here and in other studies (Figure 3A).[31,32] With regard to the maximum achievable product titer when considering the cytotoxic effects of this compound for the cells, substrate concentrations between 2.5 mM and 3 mM turned out to most suitable as up to 0.9 mM 5-bromoferulic acid (**18**) could be efficiently converted 5-bromoconiferyl alcohol (**19**) (Figure 3B). Higher

ChemBioChem 10.1002/cbic.201800673

substrate concentrations of up to 6 mM 5-bromoferulic acid (**18**) resulted in a reduced product formation, most likely due to the (probably) even more pronounced growth-inhibitory effect of substrate concentrations exceeding 2.5 mM. The observed substrate toxicity could be circumvented by stepwise addition of 5-bromoferulic acid (**18**) during the biotransformation as it was also previously demonstrated for the microbial production of *p*-coumaryl alcohol (**5**) and caffeoyl alcohol (**7**).[19] For microbial monolignol production at reactor-scale, fed-batch fermentations are a suitable option.[33] For future experiments at smaller scale, a slow-release technique could be used to avoid growth inhibiting effects of elevated phenylpropenoic acid concentrations. This technique is based on a diffusion-driven substrate release and requires a feed reservoir filled with a concentrated substrate solution.[34,35] Here, a dialysis membrane separating the reservoir from the *E. coli* cells, enables the diffusion of the substrate into the culture medium.[34,35] In principle, this approach could be also used for biotransformations at microtiter plate-scale.[36]

ChemBioChem 10.1002/cbic.201800673

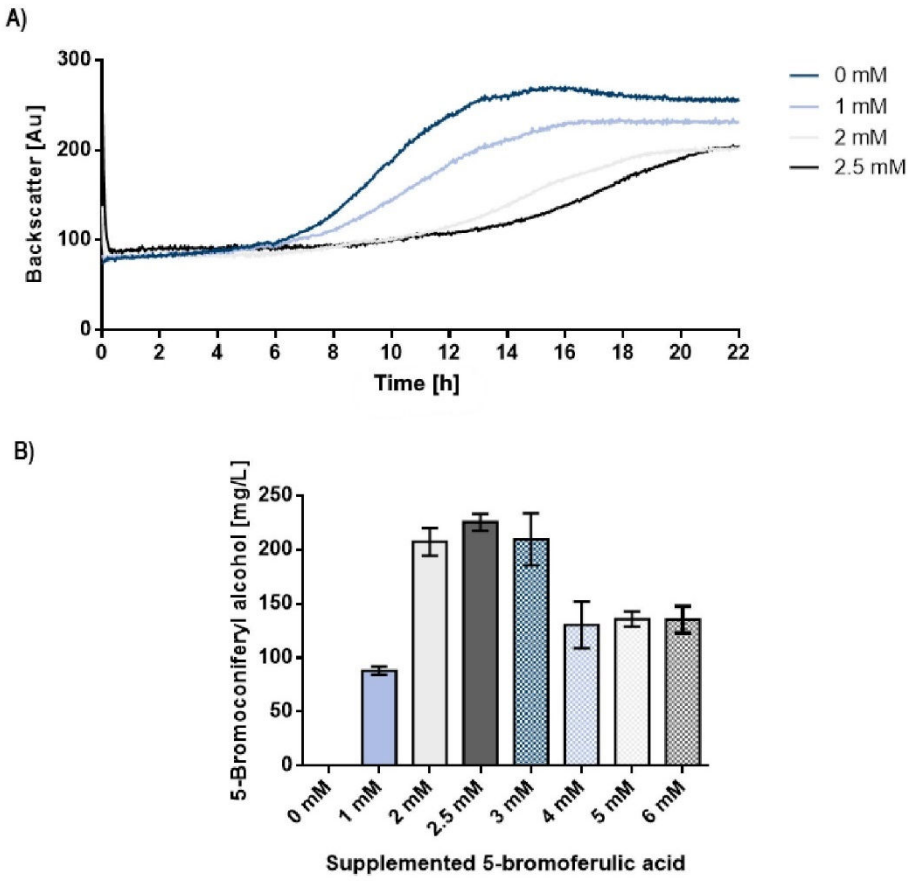

Figure 3. Impact of different 5-bromoferulic acid (**18**) concentrations on cell growth and 5-bromoconiferyl alcohol (**19**) production. A) Growth of the engineered *E. coli* strain in the presence of 5-bromoferulic acid (**18**) concentrations ranging from 0 mM to 2,5 mM B) Obtained 5-bromoconiferyl alcohol (**19**) concentrations in the presence of varying 5-bromoferulic acid (**18**) concentrations. *E. coli* BL21-Gold (DE3) *lacI*^{Q1} pALXtreme-*tal-4cl-ccr-cad* was cultivated in 900 µL LB medium with different 5-bromoferulic acid (**18**) concentrations in 48-well microtiter plates at 25 °C and 900 rpm. Heterologous gene expression was induced with 1 mM IPTG at the time point of inoculation. 5-Bromoconiferyl alcohol (**19**) concentrations were determined by HPLC. Data represents average values and standard deviations from three biological replicates.

ChemBioChem 10.1002/cbic.201800673

Conclusions

In this study, *E. coli* BL21-Gold (DE3) *lacI*^{Q1} pALXtreme-*tal-4cl-ccr-cad* was characterized with regard to the biosynthetic versatility of the heterologous monolignol pathway. In this context, it could be shown that this strain represents a suitable catalyst for the production of six naturally and four non-natural occurring monolignols. Key to the success was the relaxed substrate specificity of the enzymes within this synthetic pathway, which accept a broad range of phenylpropanoid-like compounds as substrate.

In the context of this study, microbial synthesis of the naturally occurring hydroxyconiferyl alcohol (**14**) and sinapyl alcohol (**15**) could be demonstrated for the first time. In addition, the chemically interesting monolignols 3,4,5-trimethoxycinnamyl alcohol (**17**), 5-bromoconiferyl alcohol (**19**), 2-nitroconiferyl alcohol (**21**) and the 'bicyclic' *p*-coumaryl alcohol **23** could be synthesized by this *E. coli* strain. These compounds represent interesting starting points for the synthesis of more complex plant-inspired active agents.

Experimental Section

Bacterial strains, plasmids, media and growth conditions

E. coli BL21-Gold (DE3) *lacI*^{Q1} pALXtreme-*tal-4cl-ccr-cad* was used for monolignol production.[37] The pALXtreme vector backbone was constructed from a pET-28a(+) standard vector (Merck KGaA, Darmstadt, Germany) by removing 63 % of its sequence[38]. The resulting smaller vector was originally designed to improve the transformation efficiency in the context of screening campaigns, in which the efficient cloning and transformation of large and genetically diverse libraries is required.

ChemBioChem 10.1002/cbic.201800673

Redesign of this plasmid required the genomic integration of the *lacI*^{Q1} gene from pET-vector system yielding *E. coli* BL21-Gold (DE3) *lacI*^{Q1}. Hence, pALXtreme can be only used in combination with this strain.[38] *E. coli* was cultivated aerobically in Luria Bertani (LB) medium on a rotary shaker (130 rpm) or on LB plates (LB medium with 1.5 % agar) at 37°C.[39] Where appropriate, kanamycin (50 µg/mL) was added to the medium. Growth was determined by following the optical density at 600 nm (OD_{600}).

Chemical synthesis of phenylpropenoic acids and monolignols

Cinnamic acid derivatives and cinnamyl alcohol derivatives were either commercially available or synthesized (see Supporting Information). The compounds were supplemented during microbial monolignol synthesis and used standards for HPLC-analyses.

Microbial monolignol production with *E. coli*

For monolignol production in 500 mL baffled shake flasks, 50 mL LB medium containing 2.5 mM of the respective phenylpropenoic acid substrate was inoculated with an *E. coli* over-night culture to an OD_{600} of 0.1. The culture was incubated at 37 °C and 120 rpm until an OD_{600} of 0.2 was reached. Subsequently, the cultivation temperature was decreased to 25 °C and heterologous gene expression was induced with 1 mM IPTG when an OD_{600} of 0.6 was reached. Samples were taken 17 h after IPTG addition for substrate/product analyses. All cultivations were performed in biological triplicates.

For the microbial production of monolignols at microtiter plate-scale, *E. coli* cells were cultivated using a BioLector device (m2p-labs GmbH, Germany). For this purpose, cultivations were performed in 900 µL LB medium using 48-well flower plates. These

ChemBioChem 10.1002/cbic.201800673

plates were incubated at 900 rpm and 25 °C, a humidity of 85% and a throw of ø 3 mm. When using this cultivation format, heterologous gene expression was induced with 1 mM IPTG at the time point of inoculation. All cultivations were performed in biological triplicates.

Quantification of phenylpropenoic acids and monolignols

Concentrations of phenylpropenoic acids and monolignols in cell free culture supernatants were determined by HPLC using an Agilent 1260 infinity LC device (Santa Clara, CA, USA) coupled with a DAD detector. For analyses, a mixture of water with 2 % (v/v) acetic acid (buffer A) and acetonitrile with 2 % (v/v) acetic acid (buffer B) as the mobile phases was used. LC separation was carried out using a ZORBAX Eclipse AAA (3.5 µm, 4.6 × 75 mm) column with a guard cartridge (4.6 × 12.5 mm) at 50°C. For an efficient separation, 85 % buffer A and 15 % buffer B were used for a maximum of 35 min with one additional minute as post time. Substrates and products were detected by monitoring the absorbance at a defined single wavelength (Table 2). Benzoic acid (final concentration 100 mg/L, 0.82 mM) was used as internal standard. Authentic metabolite standards were either purchased from Sigma-Aldrich (Schnelldorf, Germany) or chemically synthesized in-house. Six different concentrations of each standard dissolved in acetonitrile were measured for each calibration curve. Calibration curves were calculated based on analyte/internal standard ratios for the obtained area values.

Coniferyl alcohol extraction from culture supernatant

Culture supernatants were carefully acidified to pH 6.0 using 1 M hydrochloric acid. Subsequently, the coniferyl alcohol was extracted three times with 450 mL ethyl acetate. The combined organic layers were dried with $MgSO_4$, filtrated and the solvent

ChemBioChem 10.1002/cbic.201800673

was removed under reduced pressure. The resulting product was purified via column chromatography (*n*-pentane: ethyl acetate 60:40).

Acknowledgements

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ChemBioChem 10.1002/cbic.201800673

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2.3 Engineering the substrate specificity of a caffeic acid *O*-methyltransferase from *Medicago sativa*

Engineering the substrate specificity of a caffeic acid *O*-methyltransferase from *Medicago sativa*

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Abstract

Phenylpropanoids are a diverse group of plant natural products and are of great interest due to their health beneficial properties. Their production at a larger scale could be achieved by the functional implementation of phenylpropanoid pathways in microbes. Here, we describe the site-saturation mutagenesis of the caffeic acid *O*-methyltransferase (COMT) from *Medicago sativa* for the methylation of caffeic acid and 5-hydroxyferulic acid in whole cell biotransformations with *E. coli* strains. Resulting COMT variants were evaluated regarding their potential to catalyse the synthesis of ferulic acid and sinapic acid from caffeic acid and 5-hydroxyferulic acid, respectively. Our results showed that substitutions of residues in position 136, 162 and 172, which are located in the methoxy binding pocket, influence the substrate specificity and the productivity. A COMT variant A162S/F172S enabled the recombinant *E. coli* strain to produce 36.2 ± 0.1 mg/L ferulic acid and 62.0± 4.2 mg/L sinapic acid, which is 15 % more sinapic acid than the wild type. Substitutions to arginine in position 136 and glutamine in position 172 resulted in the exclusive production of 12.8 ± 1.9 mg/L sinapic acid. In addition, the COMT variant V135I, gave rise to 25 % increased ferulic and sinapic acid titers compared to the wild type enzyme. A second site-saturation mutagenesis approach in position 135 led to the selective ferulic acid or sinapic acid production in microtiter plate scale. Modified and characterized COMT variants obtained from this study will contribute to a better understanding of these enzymes and might prove useful for the metabolic engineering of microbes for the biotechnological production of more complex phenylpropanoid-derived compounds, in future.

Keywords

O-methyltransferases, phenylpropanoids, site-saturation mutagenesis, whole cell biotransformation, phenylpropanoid synthesis

Funding information

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Introduction

Plant-derived phenylpropanoids and phenylpropanoid-derived polyphenols are important compounds for the application as colourants, antibiotics and therapeutics (Licciardi and Underwood, 2011; Marienhagen and Bott, 2013; Mi *et al.*, 2016). For example, some polyphenols are added to processed food products to make up for the colour losses during processing, some other have antioxidant properties and reduce the risk of cancer (Guo *et al.*, 2009; Sowbhagya and Chitra, 2010; Tan *et al.*, 2011).

Due to the usually low concentrations of polyphenols in plants, their extraction from plants is not efficient (Georgiev *et al.*, 2009). Another drawback is that the plant tissue is composed of a mixture of phenylpropanoids with very similar chemical properties, which makes the downstream processing cost-intensive and elaborately (Chemler and Koffas, 2008). Moreover, plants underly several environmental factors that influence the polyphenol yield (Milder *et al.*, 2005; Zhu *et al.*, 2018).

To overcome these limitations the microbial production of phenylpropanoids and phenylpropanoid-derived polyphenols represents a good alternative to access various plant-derived compounds selectively (Milke *et al.*, 2018). Mostly plant-derived enzymes participating in the phenylpropanoid and polyphenol pathway were integrated in microbial strains to produce the desired compound. For instance, pterostilbene, kaempferol and quercetin were successfully produced by respective recombinant *Corynebacterium glutamicum* strains (Kallscheuer *et al.*, 2017). A further example is the production of resveratrol in *C. glutamicum* and *Saccharomyces cerevisiae*, which was reported to have a positive effect on cardiovascular diseases in mice (Kallscheuer *et al.*, 2016; Li *et al.*, 2016). Besides, the production of the stilbene pinosylvin was demonstrated using *Escherichia coli* as a platform organism (van Summeren-Wesenhagen and Marienhagen, 2015). In all these examples the metabolically active microorganisms were fed either with a precursor or glucose and the desired products were secreted into the cultivation broth reaching product titers varying from 10 mg/L (quercetin) to 800 mg/L (resveratrol).

Some of the enzymes participating in the phenylpropanoid pathway are known to be promiscuous regarding substrate specificity (Ferrer *et al.*, 2008), which bears the risk to produce several different compounds, which are difficult to separate (Allewell, 2012; Yoon *et al.*, 2012).

One example is the is the caffeic acid *O*-methyltransferase (COMT), which catalyses the methylation of different hydroxycinnamic acid derivatives (Inoue *et al.*, 2000; Parvathi *et al.*, 2001) like caffeic acid and 5-hydroxyferulic acid (Fig. 1).

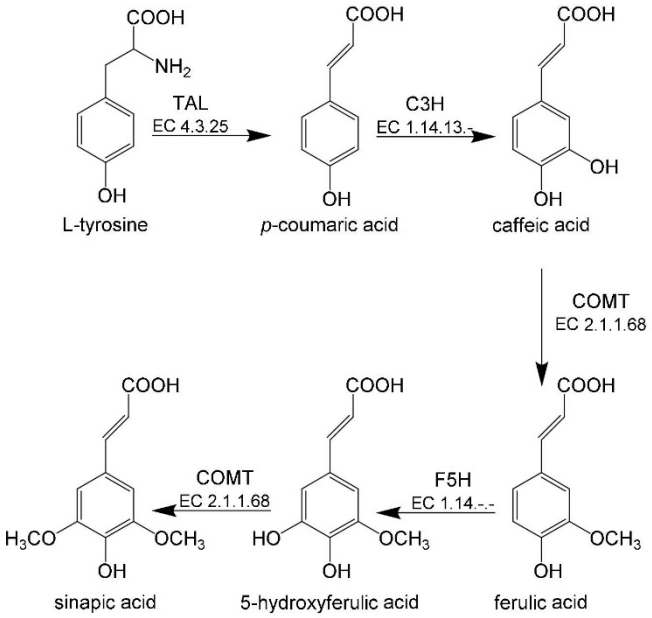

Fig. 1 Pathway of hydroxycinnamic acid derivatives. TAL: Tyrosine ammonia lyase, C3H: *p*-coumarate 3-hydroxylase, COMT: caffeic acid O-methyltransferase, F5H: ferulate 5-hydroxylase.

Methyltransferases are ubiquitous in plants and are essential for the lignin formation in cell walls (Hatfield and Vermerris, 2001). They facilitate the access to various phenylpropanoids, which serve as basis for numerous polyphenols (Schönherr and Cernak, 2013). The methyltransferase from *Medicago sativa* consists of 1098 bp coding for 365 amino acid resulting in an enzyme with 43 kDa. Its structure was analysed with X-ray crystallography, providing valuable information about reaction mechanism and substrate binding (Zubieta *et al.*, 2002). The catalytic mechanism is comparable to other plant-derived *O*-methyltransferases (Zubieta *et al.*, 2001) using *S*-adenosyl-L-methionine (SAM) as a cofactor (Fig. 2).

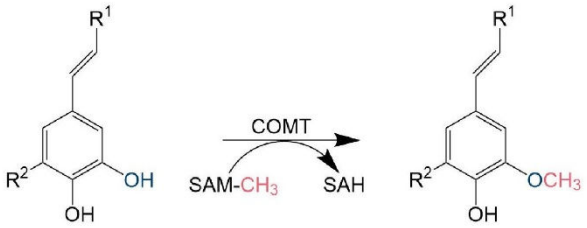

Fig. 2 *O*-Methylation of phenylpropanoids catalysed by COMT. The hydroxyl group serves as methylation site and is shown in blue. The methyl group, which is donated by *S*-adenosyl-L-methionine (SAM) is marked in red. SAH: *S*-Adenosyl-L-homocysteine; R^1: -COOH (carboxylic acid), -CO (aldehyde), -COH (alcohol); R^2: -H (proton), $-OCH_3$ (*O*-methylation).

A hydroxyl group at the aryl ring is deprotonated by a histidine at position 269, which enables the transfer of the reactive methyl group of SAM (Zubieta *et al.*, 2002). The functional enzyme consists of two subunits building up the active site. Each monomer contains a catalytic C-terminal domain, which consists of an α/β-Rossmann fold and is important to bind the cofactor SAM (Rossmann *et al.*, 1974). The transmethylation of SAM results in *S*-adenosyl-L-homocysteine (SAH). The substrate binding site next to His 269 and the cofactor is relatively large, which supports the observed promiscuous activity of COMT towards several phenylpropanoids (Zubieta *et al.*, 2001).

Until now, COMT from *M. sativa* was characterized *in vitro*, but the *in vitro* methyltransferase application bears risk regarding cost effectiveness and feasibility. The cofactor SAM is instable and expensive and the side product SAH is an inhibitor for many methyltransferases (James *et al.*, 2002). Moreover, the SAM cofactor regeneration is ATP-depended (Markham *et al.*, 1980), and therefore an *in vitro* approach is limited. Besides the implementation of an *in vitro* SAM regeneration system (Mordhorst *et al.*, 2017), whole cell biotransformations can be used to overcome these limitations.

In this study, we used COMT derived from *M. sativa* in whole cell biotransformations. We constructed a library with numerous COMT mutants and analysed these variants regarding their capability to methylate caffeic acid, a protonated phenylpropanoid, and 5-hydroxyferulic acid, an *O*-methylated phenylpropanoid, resulting in ferulic acid and sinapic acid, respectively.

Material and Methods

Plasmid construction

The *comt* gene was derived from *Medicago sativa* (GeneBank: JN850037.1) and was used for the production of ferulic acid and sinapic acid (Inoue *et al.*, 1998). The gene was codon-optimised for expression in *E. coli* and synthesized by Gene Art (ThermoFisher Scientific, Waltham, MA, USA). Oligonucleotide primers were purchased from Eurofins Genomics (Ebersberg, Germany).

An amplification of *comt* with primers 5MsEcComt_s and 5MsEcComt_as resulted in the insertion of *Hind*III and *Xho*I restriction sites and the addition of a ribosome binding site and a stop codon. This fragment and the vector pALXtreme were digested with the mentioned enzymes and ligated to pALXtreme-*comt*. The vector backbone was constructed using pET-28a (+) vector by removing 63 % of its sequence. This required the integration of *lacI*^{Q1} into the genome of *E. coli* BL21-Gold (DE3) (Blanusa *et al.*, 2010). The constructed expression plasmid was used as *comt* wild type control and as a template for library construction. Gene sequence, protein sequence and primer sequences can be found in Supplementary Material (SI, SII).

Site-saturation mutagenesis with QuikChange™ and mega primers

QuikChange™ (Stratagene, La Jolla ,CA, USA) PCR were used for the substitution of single amino acids and for the site-saturation mutagenesis at specific positions (Kunkel, 1985). All amplifications were performed with the „KOD Hot Start DNA Polymerase Kit" (ThermoFisher Scientific Waltham, MA; USA). It contains the „KOD Hot Start" DNA polymerase, which is known for its high activity and low error-rate (Takagi *et al.*, 1997).

Since the distance between the codons of interests coding for amino acids at position 135, 162 and 172 was too long for the simultaneous saturation using QuikChange™ PCR, a mega primer approach was used instead. They allow the mutation of remote codons regardless of the nucleotide distance (Zhang and Mannervik, 2013). The saturation was achieved by using NNK primers. The degenerated base N is complementary to any other base, whereas K is only complementary to guanine and thymine. This reduces the stop codons, but maintains the amino acid diversity at the same time (Dennig *et al.*, 2011). In an initial PCR step, mega primers, which cover all

desired codon positions, were generated with degenerated NNK primer. The plasmid pALXtreme-*comt* was used as template for a first PCR reaction (step 1: 95°C. 240s; step 2: 95°C, 30 s; step 3: 69,5 °C, 10 s; step 4: 72°C, 30 s; step 5: 72°C, 600 s; 24 cycles). The mega primers were used in a second PCR reaction (step 1: 95°C. 240s; step 2: 95°C, 30 s; step 3: 69,5 °C, 10 s; step 4: 72°C, 30 s; step 5: 72°C, 600 s; 20 cycles) for the amplification of pALXtreme-*comt* resulting in a mixture of plasmids with saturated codons coding for amino acids at position 135, 162 and 172. Each PCR reaction was digested with *Dpn*I at 37°C for 2 h to remove methylated wild type DNA (Arraj and Marinus, 1983) and purified using the PCR clean-up & Gel extraction Kit (Macherey & Nagel, Düren, Germany). The mutated plasmids were used to transform *E. coli* strains.

Microtiter plate screening and shake flask cultivations

For plasmid construction, *E. coli* DH10B was cultivated in LB medium or on LB agar plates at 37 °C (Sambrook and Russel, 2001). Kanamycin was added to a final concentration of 50 µg/mL to the medium. Growth was controlled by measuring the optical density at 600 nm (OD_{600}).

The screening was performed in 96 deep well plates filled with 900 µL YNB, 1 mM IPTG and 2.5 mM substrate. *E. coli* BL21-Gold (DE3) *lacI*^{Q1} (van Summeren-Wesenhagen and Marienhagen, 2015) was used as production strain. The medium was inoculated with 20 µL preculture and the plate was cultivated for 17 h at 30°C, 900 rpm and 80% humidity in a shaking incubator. Each strain was used to inoculate two medium-filled deep wells for the substrate addition of caffeic acid and 5-hydroxyferulic acid, respectively. Samples were taken at the end of cultivation for phenylpropanoid determination by HPLC. Strains producing more ferulic acid or sinapic acid were selected for further characterisation in shake flasks scale.

Cultivations to produce ferulic acid and sinapic acid were performed in YNB medium with *E. coli* BL21-Gold (DE3) *lacI*^{Q1}. Baffled shake flasks (100 mL) were filled with 10 mL YNB medium and inoculated with a preculture to an optical density of OD_{600} 0.1. The cultivation was incubated on a rotary shaker (130 rpm) at 37 °C until OD_{600} 0.2. After that, the temperature was lowered to 25 °C and the cultivation was proceeded until OD_{600} 0.6. Gene expression was induced with 1 mM IPTG (final concentration), the substrates (caffeic acid or 5-hydroxyferulic acid) were added to a final

concentration of 2.5 mM and the cultivation was continued for 17 h. Samples were taken at the end of the cultivation and the formed phenylpropanoids were analysed by HPLC (see below).

Quantification of phenylpropanoids

Phenylpropanoids in cell-free culture supernatants were quantified using a HPLC system (Agilent 1260 infinity LC device, Santa Clara, CA, USA) equipped with an UV detector. A ZORBAX Eclipse AAA (3.5 µm, 4.6 × 75 mm) column with a guard cartridge (4.6 × 12.5 mm) at 40°C was used for LC separation. A mixture of water with 2 % (v/v) acetic acid (buffer A) and acetonitrile with 2 % (v/v) acetic acid (buffer B) as the mobile phases was used for elution. For an efficient separation an isocratic gradient of 85 % buffer A and 15 % buffer B was applied for a maximum of 5 min with 1.2 mL/min and one additional minute as post time (for details see Supplementary Material, SIII). Substrates and products were detected by monitoring the absorbance at 230 nm and 320 nm, respectively. Benzoic acid was used as an internal standard with a concentration of 100 mg/L. The detection limit for phenylpropanoids was 3.9 mg/L.

Results

Optimisation of the whole cell biotransformation using wild type COMT

In initial experiments it had to be verified, that the strain *E. coli* BL21-Gold (DE3) *lacI*^{Q1} harbouring the *comt* gene under the control of the IPTG-inducible T7 promoter, is capable to methylate caffeic acid and 5-hydroxyferulic acid *in vivo* (Fig. 1). Therefore, caffeic acid was added to the cultivations (2.5 mM) in LB medium and resulted in the production of 4.0 ± 0.2 mg/L ferulic acid after 17 h. The synthesis of sinapic acid could not be shown and neither the product nor the substrate 5-hydroxyferulic acid was detectable by HPLC. Moreover, a dark reddish-brown colouring of the cultivation broth was observed. In previous studies, 7.1 mg/L ferulic acid was produced with a different set of enzymes in M9 medium, which is buffered at pH 7 using Tris/HCl (Choi *et al.*, 2011). To examine the impact of the pH on the product formation, the strain was cultivated in buffered YNB medium with initial pH values of pH 6, pH 7 and pH 8. The highest product concentrations were observed for sinapic acid at pH 7 (Fig. 3).

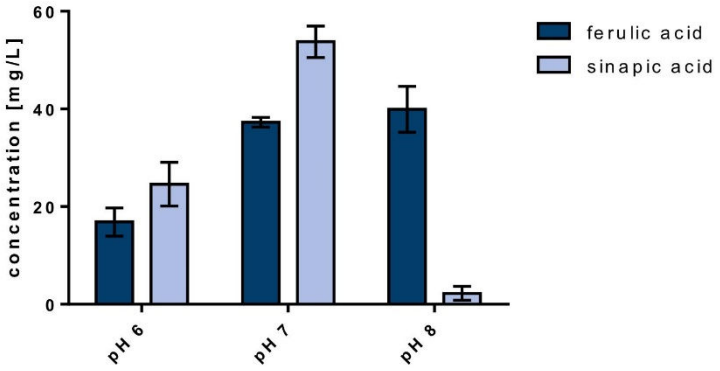

Fig. 3 Ferulic acid and sinapic acid concentration in YNB medium with different pH values. *E. coli* BL21-Gold (DE3) *lacI*^{Q1} pALXtreme-*comt* was cultivated in 10 mL YNB medium with different pH values in 100 mL baffled shake flasks at 25 °C and 130 rpm. Heterologous gene expression was induced with 1 mM IPTG at OD_{600} 0.6 and caffeic acid or 5-hydroxyferulic acid was added to a final concentration of 2.5 mM. Ferulic acid and sinapic acid concentrations were determined with HPLC. Data represents average values and standard deviations from three biological replicates.

As demonstrated in Fig. 3, there is clear pH dependency regarding ferulic acid and sinapic acid production using the recombinant *E. coli* strain expressing the wild type COMT gene. At pH 7 the yield for both products in the culture supernatant was maximal: 37.3 ± 0.8 mg/L ferulic acid and 53.8 ± 2.6 mg/L sinapic acid. At pH 6, the ferulic acid and sinapic acid production was also detected, but with lower titres. Noticeable were the product concentrations in cultivations at pH 8. The ferulic acid titre of 39.9 ± 3.8 mg/L was comparable with the titre at pH 7, but the sinapic acid concentration of 2.2 ± 1.2 mg/L was rather low. Based on these results, the screening will be performed in YNB medium buffered at pH 7 using Tris/HCl.

Selection of residues for site-saturation mutagenesis and library construction

The crystal structure of COMT derived from *Medicago sativa* enabled further investigations regarding substrate binding (Zubieta *et al.*, 2002). The α,β-unsaturated carboxylic acid moiety of the substrate is stabilized within the enzyme by two α-helices (Fig. 4).

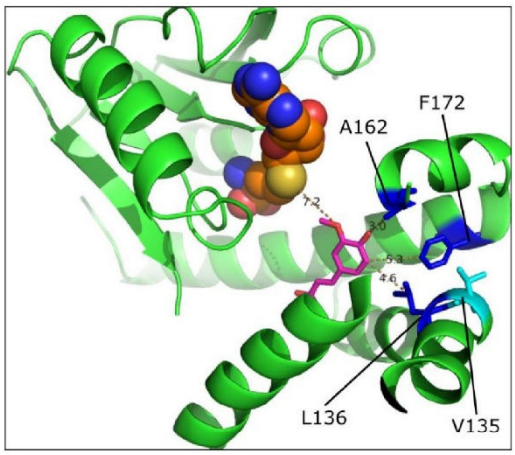

Fig. 4 View into active centre of COMT. S-Adenosyl-L-homocysteine (SAH) is shown as space-filling structure (yellow: sulphur, red: oxygen, blue: nitrogen). Ferulic acid, the product of the *O*-methylation, is presented in magenta. Amino acid residues with postulated impact on substrate specificity are coloured in blue. Dashed lines assign the distance in Å.

The functional group of the substrate side chain is stabilised by hydrogen bonds to H183 and N131. This orientation hinders the methylation in *para* position of the aromatic ring and contributes to the substrate preference of the cinnamic aldehyde derivatives over the respective cinnamic alcohol derivatives and the cinnamic acid derivatives (Osakabe *et al.*, 1999; Parvathi *et al.*, 2001). The side chains of L136, A162 and F172 are responsible for the substrate preference 5-hydroxyconiferyl aldehyde > caffeoyl aldehyde > 5-hydroxyferulic acid > caffeic acid (Bugos *et al.*, 1992; Inoue *et al.*, 2000; Parvathi *et al.*, 2001). Therefore, these positions were subjected to site-saturation mutagenesis to construct a library for the enhanced ferulic acid and sinapic acid production. Sequencing of ten clones helped to estimate the library diversity. All sequenced *comt* variants showed at least one nucleotide substitution in each addressed codon (Fig. 5 A).

B

	Position		
	136	162	172
Wild type	L	A	F
Variant 1	A	T	V
Variant 2	R	M	G
Variant 3	G	A	R
Variant 4	E	E	I
Variant 5	R	L	C
Variant 6	R	STOP	M
Variant 7	G	L	Q
Variant 8	G	F	G
Variant 9	R	V	A
Variant 10	G	L	Q

Fig. 5 Sequence alignment of different COMT mutants and amino acid substitutions. (A) The first sequence indicates the wild type; the following lines are selected mutants. The nucleotide sequence showed unique substitutions coding for amino acids at position 136, 162 and 172. (B) The amino acid substitutions indicated a sufficient variation in amino acid residues. Letters stand for the one-letter amino acid code.

These substitutions resulted in amino acid variations at each position and multiple substitutions in one variant (Fig. 5B).

In previous studies it was shown that a substitution of A162 by threonine leads to higher specificity towards 5-hydroxyferulic acid (Zubieta *et al.*, 2002). Therefore, this variant was constructed as a control for higher sinapic acid production.

Screening of COMT variants

Site-saturation mutagenesis of *comt*-encoding residues in position 136, 162 and 172 was performed to construct variants with enhanced ferulic acid production or sinapic production. Each clone was used to inoculate two deep wells for the substrate addition of caffeic acid and 5-hydroxyferulic acid, respectively. The product titres of both cultivations were compared and evaluated regarding the capability of producing ferulic acid or sinapic acid (Fig. 6).

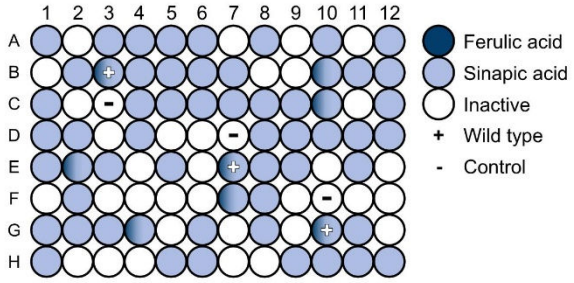

Fig. 6 Schematic representation of a microtiter plate screening for strains producing different COMT variants. Each well represents a different COMT variant produced by *E. coli* BL21-Gold (DE3) *lacI*^{Q1}. The strains were cultivated twice, and cultivation conditions differed in substrate addition (caffeic acid or 5-hydroxyferulic acid). A well was marked in dark blue, when the strain produced ferulic acid, a light blue well indicated sinapic acid production. Ferulic acid and sinapic acid were determined by HPLC. White: inactive variants, +: wild type COMT variant, -: control without inoculation.

Despite the COMT variants, the microtiter plate also contained three not inoculated wells as controls and three wells inoculated with a strain coding for the COMT wild type variant. This strain produced 5.2 ± 0.2 mg/L ferulic acid and 5.7± 2.3 mg/L sinapic acid and was used as a reference for the concentrations produced by strains harbouring different COMT variants.

Overall, 1080 COMT variants were tested regarding the produced concentrations of ferulic acid and sinapic acid. On average, one third of all tested variants were inactive, two third produced exclusively sinapic acid and 4 % were capable to produce ferulic acid and sinapic acid. These first results were used to select promising COMT variants for further characterisation in shake flask scale (10 mL). Moreover, the nucleotide sequence of these variants was determined to have a closer look at the corresponding amino acid substitutions.

The shake flasks cultivations revealed that the amino acid substitution at the selected position influenced productivity and substrate specificity. The recombinant strain containing the wild type COMT produced 37.3 ± 1.0 mg/L ferulic acid and 53.8 ± 3.2 mg/L sinapic acid (Fig. 7).

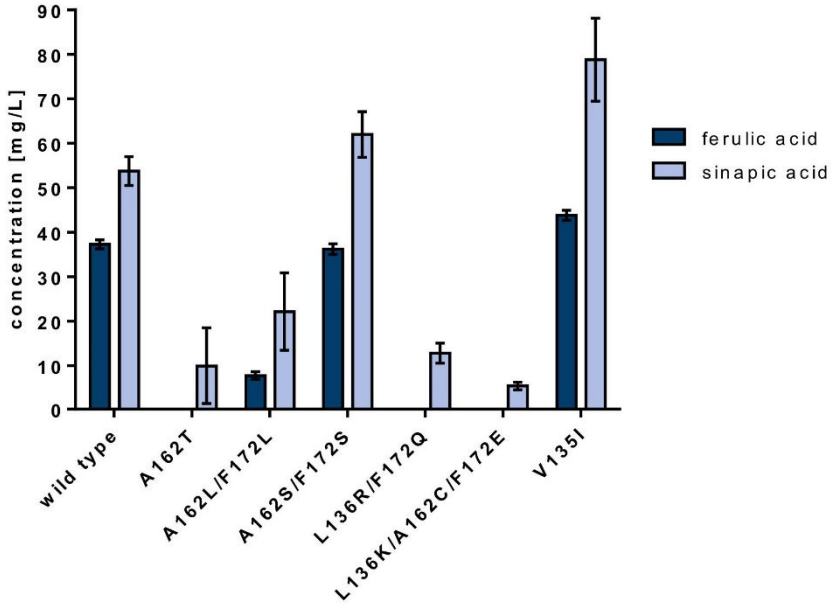

Fig. 7 Concentrations of ferulic acid and sinapic acid produced with different *E. coli* BL21-Gold (DE3) *lacI*^{Q1} strains expressing various COMT variants. Strains were cultivated in 10 mL YNB medium in 100 mL baffled shake flasks at 25 °C and 130 rpm. Heterologous gene expression was induced with 1 mM IPTG at OD_{600} 0.6 6 and caffeic acid or 5-hydroxyferulic acid was added to a final concentration of 2.5 mM. Ferulic acid and sinapic acid were determined by HPLC. Data represent average values and standard deviations from three biological replicates.

A variant with a A162S/F172S substitution produced 36.2 ± 0.1 mg/L ferulic acid and 62.0 ± 4.2 mg/L sinapic acid, which is comparable to the wild type for ferulic acid, but an increase of 15 % for the sinapic acid production. Three strains containing COMT variants with different amino acid substitutions produced exclusively sinapic acid. *E. coli* strains containing the COMT double variant with a L136R/F172Q substitution gave 12.8 ± 1.9 mg/L sinapic acid, which was the highest observed titre among those strains exclusively producing sinapic acid. The second best result was obtained with the previously published control variant A162T (Zubieta *et al.*, 2002), which produced 9.9 ± 7.0 mg/L sinapic acid. In general, it could be seen that the productivity is reduced with the quantity of amino acid substitutions.

During the course of the library screening, an *O*-methyltransferase from *Rauwolfia Serpentina* (*Rs*OmtIII) was studied, which has a higher specificity towards caffeic acid *in vitro* (Wiens and Luca, 2016). Although a sequence alignment of *Rs*OmtIII and *Ms*COMT showed a sequence identity of 75%, all previously mentioned amino acids in COMT from *M. sativa* in position 136, 162 and 172 were conserved (Fig. 8).

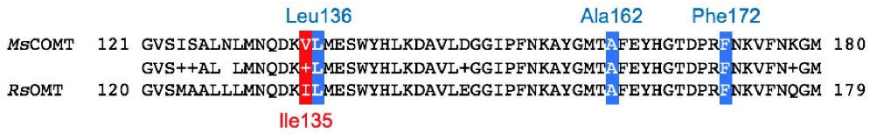

Fig. 8 Amino acid sequence alignment of two different COMT variants. The upper variant is derived from *M. sativa*, the lower variant OMTIII is derived from *Rauwolfia serpentine*. The amino acids, as part of the methoxy binding pocket, are conserved at the postulated important residues in position 136, 162 and 172 (blue). An adjacent substitution of V135 to isoleucine (red) could also have an impact on substrate binding (compare Fig. 4).

The substitution of V135 to isoleucine was the only alteration adjacent to the other positions (compare Fig. 4) and could be the reason for the different substrate specificity. We constructed a strain producing a COMT variant with this substitution and used it in whole cell biotransformations. As a result, 43.8 ± 0.9 mg/L ferulic acid and 78.8 ± 7.6 mg/L sinapic acid were produced, which is an increase of 17 % and 46 % in contrast to the wild type (Fig. 7). We postulated, that a different amino acid substitution at this position could result in even higher titres and used the previously found variants A162S/F172S and L136R/F172Q for site-saturation mutagenesis at position 135.

Screening of the sublibraries

Due to their substrate specificity and the resulting productivity in the previous studies (Fig. 7) the following COMT variants were chosen for site-saturation mutagenesis: A162S/F172S for increased ferulic acid production and L136R/F172Q for the selective sinapic acid production. After library construction and transformation of *E. coli* BL21-Gold (DE3) *lacI*^{Q1}, the clones were used to inoculate two deep wells for the substrate addition of caffeic acid and 5-hydroxyferulic acid, respectively. The product

concentrations of both cultivations were compared and evaluated regarding the production of ferulic acid and sinapic acid, respectively (Fig. 9).

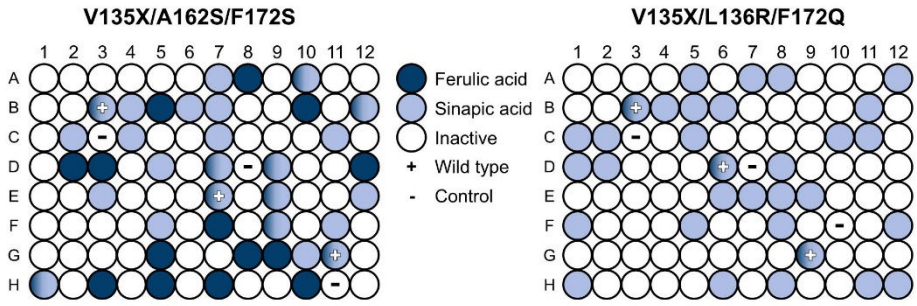

Fig. 9 Schematic representation of a microtiter plate screening for strains expressing different COMT variants. Each well represents a different COMT variant produced by *E. coli* BL21-Gold (DE3) *lacI*^{Q1} sublibraries. The strains were cultivated twice, and cultivation conditions differed in substrate addition (caffeic acid or 5-hydroxyferulic acid). Left: Site-saturation mutagenesis of COMT variant A162S/F172S at position 135. Right: Site-saturation mutagenesis of COMT variant L136R/F172Q at position 135. A well was marked in dark blue, when the strain produced ferulic acid, a light blue well indicated sinapic acid production. Ferulic acid and sinapic acid were determined by HPLC. White: inactive variants, +: wild type COMT variant, -: control without inoculation.

Saturation mutagenesis of COMT variant A162S/F172S at position 135

The screening of a total of 90 clones of the sublibrary revealed 28 active and 55 inactive variants. Moreover, 14 variants showed a selective ferulic acid production (Fig. 9). For further characterisation, the COMT nucleotide sequences of these variants were determined. The best variant among the others had a substitution at position 135 to threonine and produced exclusively ferulic acid (6.9 mg/L). Other interesting candidates had also substitutions to threonine or arginine, but with a different codon usage.

Saturation mutagenesis of COMT variant L136R/F172Q at position 135

Besides variant A162S/F172S, variant L136R/F172Q was also subjected to site-saturation mutagenesis at position 135 due to selective sinapic acid production. The

microtiter plate screening of a total of 90 clones disclosed that 52 variants were inactive, and 29 variants produced exclusively sinapic acid (Fig. 9). Gene sequencing revealed, that the highest sinapic acid concentrations were achieved by variants with substitutions to serine (8.1 mg/L) and glycine (8.0 mg/L). Other interesting candidates had substitutions to glutamine and leucine. All variants produced exclusively sinapic acid.

Discussion

O-methyltransferases play an important role for the production of phenylpropanoids (Marienhagen and Bott, 2013). Motivated by using whole cell biotransformations for a cost-efficient cofactor regeneration, we used site-saturation mutagenesis to increase the ferulic acid and sinapic acid production by COMT from *Medicago sativa*.

At the beginning, suitable cultivation conditions had to be established and YNB medium with different pH values was tested. In contrast to cultivations at other pH values, cultivations at pH 8 resulted in rather low sinapic acid concentrations (2.2 ± 1.2 mg/L). A closer look at substrate concentrations at the end of cultivation revealed, that only 21.5 ± 10.3 mg/L 5-hydroxyferulic acid were found in culture supernatants. This indicated that the pH value did not influence productivity, but substrate degradation. In previous studies it was shown, that plant-derived phenolic compounds underly a chemical degradation under mild alkaline conditions (Friedman and Jürgens, 2000). Especially compounds with several hydroxyl groups at the aryl ring react to instable quinone intermediates and are finally oxidised in the presence of air to diketo derivatives and other degradation products (Friedman and Jürgens, 2000). This could also be an explanation for the dark reddish-brown colouring of the cultivation broth.

The simultaneous saturation of three codons with NNK primers results in 32,768 possible gene variants coding for 8,000 enzyme variants. This implies that 98,160 variants needed to be analysed to cover 95 % of all possible gene sequences (Firth and Patrick, 2008). The simultaneous saturation of three codons allows for the elucidation of synergistic effects, which might have a positive effect on product formation (Shivange *et al.*, 2009). Nevertheless, only few variants with higher ferulic

acid production were found in microtiter plate-based screening, which limited the selection of variants. This limitation could be overcome by using a high-throughput screening approach. For example, fluorescence-activated cell sorting (FACS) in connection with biosensors can help to screen 70,000 variants within seconds (Yang and Withers, 2009; Marienhagen and Bott, 2013). In contrast to that, the saturation of one codon in the sublibraries results in 32 possible gene variants coding for 20 amino acids. Therefore, the screening of variants in a 96 deep well plate is sufficient to cover more than 95 % of all possible variants.

In general, the product concentration in cultivations in deep well plates were lower compared to cultivations in shake flasks. For example, the strain producing the wild type COMT variant yielded 5.2 ± 0.2 mg/L ferulic acid and 5.7± 2.3 mg/L sinapic acid in deep well plates. In shake flasks, 37.3 ± 0.8 mg/L ferulic acid and 53.8 ± 2.6 mg/L sinapic acid were detectable. One difference was, that gene expression was induced in deep well plate cultivations directly from the beginning of the cultivation and not at OD_{600} 0.6, which was the case for shake flasks cultivations. Consequently, the strain needed more energy for the synthesis of recombinant protein and less energy was available for biomass production. This causes a longer lag phase at the beginning of the cultivation (Donovan *et al.*, 1996), which might have an influence on product formation. The slightly higher cultivation temperature during deep well plate cultivation, 30 °C compared to 25 °C, could result in the degradation of 5-hydroxyferulic acid (Friedman and Jürgens, 2000). Moreover, the oxygen transfer rate, the cell growth and as a result the productivity of cultivations in microtiter plates and shake flasks can differ hugely (Duetz, 2007). These differences are caused by unfavourable cultivation parameters like high filling volumes and low shaking frequencies (Losen *et al.*, 2004; Zimmermann *et al.*, 2006). Adjusting the cultivation conditions can lead to a better comparability of screening and production results. Moreover, the gained knowledge can contribute to even higher ferulic acid and sinapic acid concentrations due to optimised cultivation conditions in shake flasks (Wewetzer *et al.*, 2015).

In the microtiter plate screening and in subsequent shake flasks experiments, some COMT variants indicated the selective production of sinapic acid, although with relatively low productivity (Fig. 7). Considered that sinapic acid was produced in excess relative to ferulic acid, the production of the latter could not be excluded and might not be detected since its concentration was below the detection limit (3.9 mg/L).

Inspection of the COMT active site revealed that the amino acids at position 136 and 172 are closer to the C5 atom of the substrate's aryl ring (Fig. 4). At this position, the substrate caffeic acid harbours a proton, whereas 5-hydroxyferulic acid contains a hydroxyl group, which makes it larger, more polar and able to form hydrogen bonds. In the library, mutants were found with several amino acid substitutions in these positions. The substitution of the amino acids by more polar amino acids like arginine, glutamine or glutamic acid led to reduced ferulic acid concentrations and an increased specificity towards sinapic acid production. This indicated that the interaction in the active site between the polar residues of these amino acids with the polar substrate 5-hydroxyferulic acid might be the reason for the increased specificity towards sinapic acid. Similar observations have been made with conversion of various cinnamic acid derivates to their corresponding CoA esters by a 4-coumarate:CoA ligase (Schneider *et al.*, 2003). Substitutions from an aliphatic (L) to a positive charged amino acid (K,R) in position 136 and from an aromatic (F) to a negatively charged amino acid (E) in position 172 were also observed. Both are quite frequent in protein active or binding sites and might cause the higher 5-hydroxyferulic acid specificity in contrast to the wild type (Betts and Russell, 2007).

The highest product concentrations were detected with a variant comprising an amino acid substitution at position 135 from valine to isoleucine (ferulic acid: 43.8 ± 0.9 mg/L, sinapic acid: 78.8 ± 7.6 mg/L), which is an increase of 25 % compared to the wild type. This residue is not located in the immediate vicinity of the substrate and a direct impact on the substrate binding pocket formation is unlikely. However, this substitution could influence the folding of the α-helix, which is adjacent to the substrate binding pocket and includes the residue at position 136 (Fig. 4). Similar second shell interactions have already been described earlier (Lingen *et al.*, 2002).

During sublibrary screening with COMT L136R/F172Q as a template, a variant was found with a substitution at position 135 to serine. This variant produced exclusively sinapic acid. Serine is a polar amino acid and is likely to interact with the more polar substrate 5-hydroxyferulic acid resulting in sinapic acid production (Betts and Russell, 2007).

Conclusion

Aim of this study was engineering the caffeic acid *O*-methyltransferase (COMT) from *Medicago sativa* for increased methylation of caffeic acid and 5-hydroxyferulic acid in whole cell biotransformations. Modified COMT variants from this site-saturation mutagenesis approach were evaluated regarding their capabilities to synthesize ferulic acid and sinapic acid from their respective precursor molecules by cultivation experiments with recombinant *E. coli* strains. These experiments showed, that amino acid substitutions at positions 136, 162 and 172 located in the methoxy binding pocket, increase the productivity and changed the substrate specificity towards caffeic acid and 5-hydroxyferulic acid. A COMT variant with a serine at position 162 and 172 enabled the recombinant *E. coli* strain to produce sinapic acid with a titre of 62.0 ± 4.2 mg/L, 15 % more than the wild type, in whole cell biotransformations. The variant L136R/F172Q produced exclusively 12.8 ± 1.9 mg/L sinapic acid. In addition, a COMT variant with a valine at position 135 was identified, which had 25 % increased ferulic and sinapic acid titres. A site-saturation of interesting variants in position 135 resulted in the selective production of ferulic acid or sinapic acid in microtiter plates. Screening of the remaining variants, maybe taking even more amino acid residues into consideration for further studies. Modified and characterized COMT variants obtained from this study will contribute to a better understanding of these enzymes and might prove useful for the metabolic engineering of microbes for the biotechnological production of more complex phenylpropanoid-derived compounds, in future.

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4 Appendix

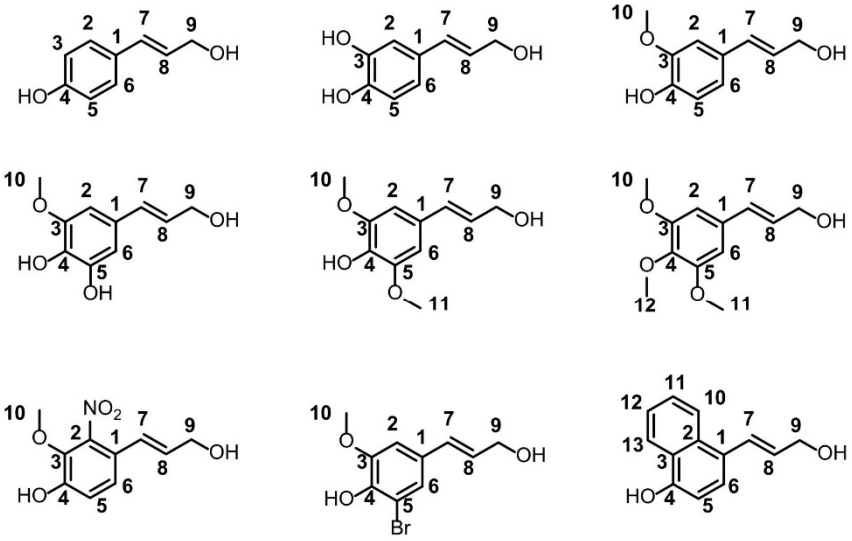
4.1 Supplementary material "Microbial production of natural and non-natural monolignols with *Escherichia coli*"

List of contents

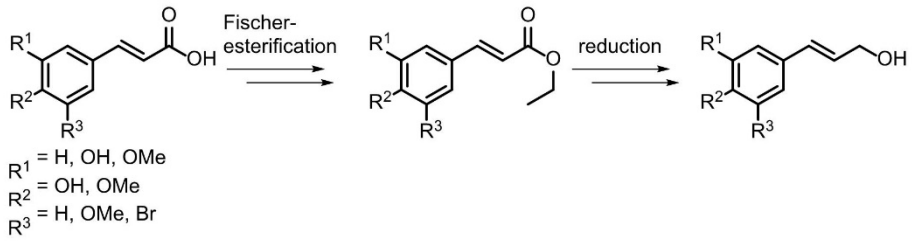
General syntheses

General: All reagents were used as purchased from commercial suppliers without further purification. Petroleum ether, *n*-pentane and ethyl acetate for column chromatography were distilled before usage. Brine refers to a saturated solution of NaCl in deionized water. Microwave reactions were performed in a CEM Discover system (SN: DU8708), equipped with an CEM Intelligent explorer (SN: NX2069). Flash column chromatography was performed on silica gel 60, particle size 40–63 µm (230–240 mesh). Absorbance measurements were conducted using an UV-160 spectrophotometer. ^{1}H and ^{13}C-NMR spectra were recorded on an Advance/DRX 600 nuclear magnetic resonance spectrometer (Bruker) at ambient temperature in $CDCl_3$ or DMSO-d_6 at 600 and 151 MHz, respectively. The chemical shifts are given in ppm relative to the solvent signal [^{1}H: δ ($CHCl_3$) = 7.26 ppm], [^{13}C: δ ($CDCl_3$) = 77.2 ppm], [^{1}H: δ (DMSO-d_6) = 2.50 ppm], [^{13}C: δ (DMSO-d_6) = 39.5 ppm], [^{1}H: δ (Acetone-d_6) = 2.05], [^{13}C: δ (Acetone-d_6) = 29.8 ppm]. NMR signals were assigned by means of H-COSY-, HSQC- and HMBC-experiments and coupling constants J are given in Hz. Chiral HPLC measurements were performed on a Dionex system equipped with a pump with a gradient mixer and devolatilizer included a WPS-3000TSL autosampler and a DAD-3000 UV-detector. Chiralpak IA column (250 mm×4.6 mm, Daicel) and a mixture of *n*-heptane/2-propanol (70:30) as solvent was used applying a flow rate of 0.5 mL/min^{-1} at r.t. Samples were dissolved in degassed *n*-heptane: 2-propanol 2:1.

Synthesis of reference alcohols



Method A



Fischer esterification

The acid (1 mmol) was solved in 2.7 mL of ethanol inside a microwave reaction tube. One drop of conc. sulfuric acid was added. The solution was then heated to 95 °C using a microwave for 30-90 min. The reaction was monitored using TLC (thin layer chromatography). After complete conversion the solution was diluted with ethyl acetate and then washed with water, followed by washing with brine. The organic phase was dried with $MgSO_4$, filtered and concentrated to

give the crude product. If necessary, the product was purified using column chromatography (petroleum ether:ethyl acetate).

TBS-Protection of ethyl (*E*)-3-(3,4-dihydroxyphenyl)acrylate (24)

The **ethyl (*E*)-3-(3,4-dihydroxyphenyl)acrylate** (24) (1.1 mmol) was solved in 7 mL dichloromethane. *tert*-Butyldimethylchlorosilane (2.5 eq., TBS-Cl) and *N,N*-ethyldiisopropylamine (3.5 eq.) were added to the stirring solution. The solution was stirred for 22 h. After complete conversion, observed by TLC, 3 mL of dichloromethane were added. The resulting solution was added to 5 mL of water. The two-phase system was washed with 10 mL brine. After phase separation the organic phase was dried with $MgSO_4$, filtered and concentrated to give pure product.[1]

4

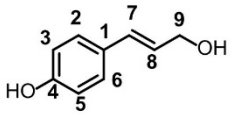
Ester reduction using DiBAl-H

Diisobutylaluminium hydride (DiBAl-H, 2.5 eq., 1 additional equivalent was added per hydroxyl group) solution (1 M in dichloromethane) was diluted with 2.3 mL tetrahydrofuran. The solution was stirred and cooled to -20 °C. The ester (3 mmol) was solved in 2.3 mL tetrahydrofuran and added dropwise over 30 min. The reaction was stirred for 1 h at -20 °C and monitored using TLC. After complete conversion, the excess DiBAl-H was quenched using ethyl acetate at 0 °C. Half-saturated NaK-tartrate solution was added. After phase separation, the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried with $MgSO_4$ and subsequently filtered. Pure product could be isolated after removal of the solvent under reduced pressure.[2]

Deprotection of (*E*)-3-(3,4-bis((*tert*-butyldimethylsilyl)oxy)phenyl)-prop-2-en-1-ol

(*E*)-3-(3,4-bis((*tert*-butyldimethylsilyl)oxy)phenyl)-prop-2-en-1-ol (0.5 mmol) was solved in 30 mL tetrahydrofuran and 1.1 mL acetic acid. The solution was cooled to 0 °C and *tetra*-butylammonium fluoride solution (2.5 eq., 1 mol/L in tetrahydrofuran) was added. Afterwards the reaction was stirred for 2 h at 0 °C. After completion the volume was reduced to 50%, which resulted in precipitation of a yellow solid. This solid was collected and washed with chloroform, until the yellow colour disappeared. Pure (*E*)-3-(3,4-dihydroxy)phenyl)-prop-2-en-1-ol (7) could be isolated after drying.[3]

(*E*)-3-(4-Hydroxy)phenyl)-prop-2-en-1-ol (*p*-coumaryl alcohol) (5)



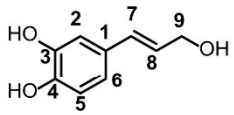
Yield: 35%

5

^{1}H-NMR ($CDCl_3$, 600 MHz) δ [ppm]: 4.16-4.20 (m, 2 H, 9-H); 6.20 (dt, $^{3}J_{8,7}$ = 15.8 Hz, $^{3}J_{8,9}$ = 5.6 Hz, 1 H, 8-H); 6.51 (d, $^{3}J_{7,8}$ = 15.9 Hz, 1 H, 7-H); 6.79 (d, $^{3}J_{3/5,2/6}$ = 8.2 Hz, 2 H, 3-H and 5-H); 7.27 (d, $^{3}J_{2/6,3/5}$ = 8.2 Hz, 2 H, 2-H and 6-H)

^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 62.6 (s, C-9); 115.3 (s, C-3 and C-5); 127.0 (s, C-8); 127.5 (s, C-2 and C-6); 128.9 (s, C-1); 129.2 (s, C-7); 156.9 (s, C-4)

(*E*)-3-(3,4-Dihydroxy)phenyl)-prop-2-en-1-ol (caffeoyl alcohol) (7)

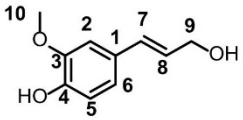


Yield: 43%

^{1}H-NMR ($CDCl_3$, 600 MHz) δ [ppm]: 4.04 (dd, $^{3}J_{9,9\text{-OH}}$ = 5.4 Hz, $^{4}J_{9,7}$ = 1.7 Hz, 2 H, 9-H); 4.73 (d, $^{3}J_{9\text{-OH},9}$ = 5.5 Hz, 1 H, 9-O*H*); 6.03 (dt, $^{3}J_{7,8}$ = 15.9 Hz, $^{3}J_{9,8}$ = 5.5 Hz, 1 H, 8-H); 6.03 (d, $^{3}J_{7,8}$ = 15.8 Hz, 1 H, 7-H); 6.67-6.64 (m, 2 H, 6-H and 5-H); 6.80 (d, $^{3}J_{2,6}$ = 1.4 Hz, 1 H, 2-H); 8.85 (s, 1 H, 3-OH); 8.92 (s, 1 H, 4-O*H*)

^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 61.7 (s, C-9); 113.0 (s, C-2); 115.6 (s, C-5); 117.9 (s, C-6); 127.0 (s, C-8); 128.4 (s, C-1); 129.0 (s, C-7); 145.0 (s, C-4); 145.3 (s, C-3)

(*E*)-3-(4-Hydroxy-3-methoxy)phenyl)-prop-2-en-1-ol (Coniferyl alcohol) (8)



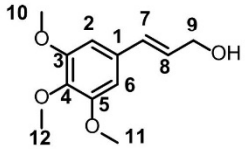
Yield: 90%

^{1}H-NMR ($CDCl_3$, 600 MHz) δ [ppm]: 3.82 (s, 1 H, 9-OH); 3.87 (s, 3 H, 10-H); 4.19 (dd, $^{3}J_{9,8}$ = 5.9 Hz, $^{4}J_{9,7}$ = 1.7 Hz, 2 H, 9-H); 5.63 (s, 1 H, 4-OH); 6.23 (dt, $^{3}J_{8,7}$ = 15.8 Hz, $^{3}J_{8,9}$ = 6.0 Hz, 1 H, 8-H); 6.54 (dt, $^{3}J_{7,8}$ = 15.8 Hz, $^{4}J_{7,9}$ =1.6 Hz,

1 H, 7-H); 6.77 (d, $^{3}J_{5,6}$ = 8.1 Hz, 1 H, 5-H); 6.86 (dd, $^{3}J_{6,5}$ = 8.1 Hz, $^{4}J_{6,2}$ = 2.0 Hz, 1 H, 6-H); 7.06 (d, $^{4}J_{2,6}$ = 2.0 Hz, 1 H, 2-H)

^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 56.0 (s, C-10); 63.9 (s, C-9); 108.5 (s, C-2); 114.6 (s, C-5); 120.4 (s, C-6); 126.3 (s, C-8); 129.3 (s, C-1); 131.5 (s, C-7); 145.7 (s, C-3 or C-4); 146.77 (s, C-4 or C-3)

(*E*)-3-(3,4,5-Trimethoxy)phenyl)-prop-2-en-1-ol(3,4,5-trimethoxycinnamoyl alcohol) (17)

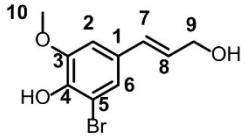


Yield: 46%

^{1}H-NMR ($CDCl_3$, 600 MHz) δ [ppm]: 3.84 (s, 3 H, 12-H); 3.87 (s, 6 H, 10-H and 11-H); 4.32 (m, 2 H, 9-H); 6.29 (dt, $^{3}J_{8,7}$ = 15.8 Hz, $^{3}J_{8,9}$ = 7.1 Hz, 1 H, 8-H); 6.54 (d, $^{3}J_{7,8}$ = 15.8 Hz, 1 H, 7-H); 6.61 (s, 2 H, 2-H and 6-H)

^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 56.2 (s, C-10 and C-11); 61.1 (s, C-9); 63.8 (s, C-12); 103.7 (s, C-2 and C-6); 128.2 (s, C-8); 131.3 (s, C-7); 132.6 (s, C-1); 138.0 (s, C-4); 153.4 (s, C-3 and C-5)

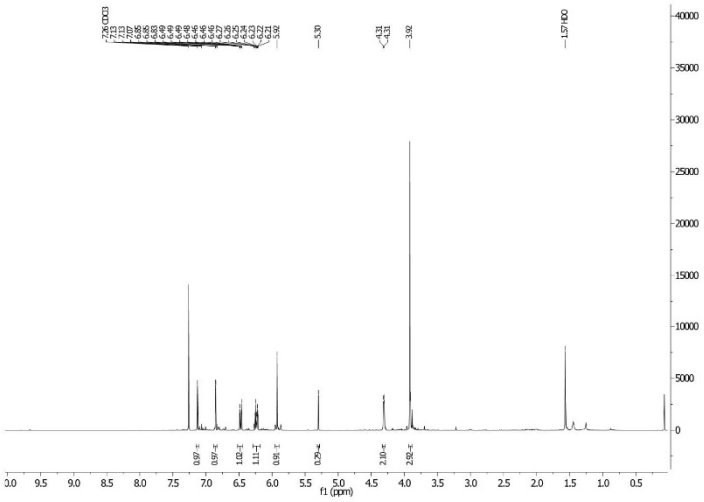
(*E*)-3-(5-Bromo-4-hydroxy-3-methoxy)phenyl)-prop-2-en-1-ol (5-bromo-coniferyl alcohol) (19)



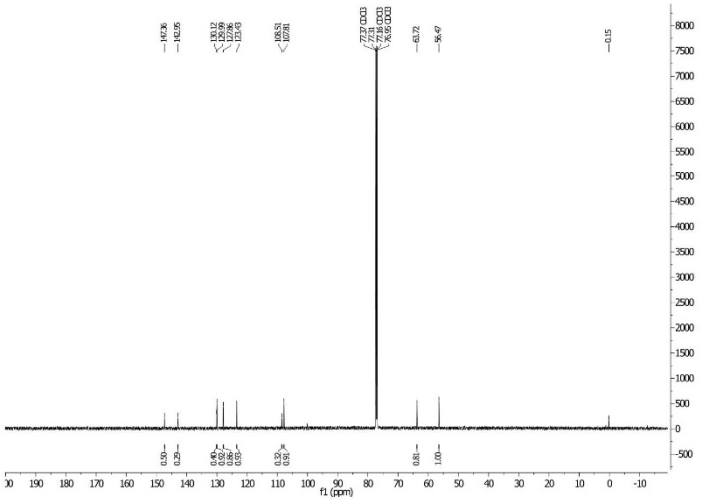
Yield: 80%

^{1}H-NMR ($CDCl_3$, 600 MHz) δ [ppm]: 3.92 (s, 3 H, 10-H); 4.31 (d, $^{3}J_{9,8}$ = 5.7 Hz, 2 H, 9-H); 5.30 (s, 1 H, 9-O*H*); 5.92 (s, 1 H, 4-O*H*); 6.23 (dt, $^{3}J_{8,7}$ = 15.8 Hz, $^{3}J_{8,9}$ = 5.8 Hz, 1

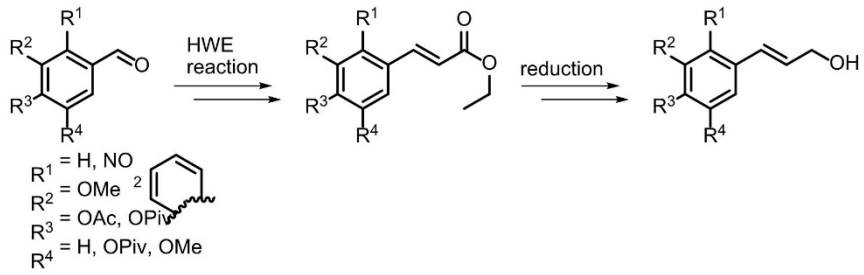
H, 8-H); 6.47 (d, $^{3}J_{7,8}$ = 15.8 Hz, 1 H, 7-H); 6.85 (d, $^{4}J_{6,2}$= 1.8 Hz, 1 H, 6-H); 7.13 (d, $^{4}J_{2,6}$= 1.8 Hz, 1 H, 2-H)



^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 56.5 (s, C-10); 63.7 (s, C-9); 107.8 (s, C-6); 108.5 (s, C-4); 123.4 (s, C-2); 127.9 (s, C-8); 123.0 (s, C-7); 130.1 (s, C-5); 142.9 (s, C-1); 147.4 (s, C-3)



Method B



Protection of phenols with acetic anhydride

The aldehyde (6 mmol) together with 0.2 eq. dimethylaminopyridine was solved in 6.4 mL of dichloromethane. Triethylamine (3 eq. per hydroxyl group) and acetic anhydride (1.2 eq. per hydroxyl group) were added to the stirred solution. The reaction was stirred for 1 h at 0 °C, followed by 4 h at 24 °C. After completion the reaction was quenched using water. The phases were then separated and the organic phase was washed with saturated $NaHCO_3$-Solution. Water was removed by washing with brine and through $MgSO_4$ addition. Following filtration, the solvent was removed and pure product could be isolated.[4]

HWE reaction

Triethyl phosphonoacetate (1.7 eq.) was solved in 1.6 mL tetrahydrofuran and the solution cooled to 0 °C. NaH (1.7 eq., 60% suspension in mineral oil) were added in multiple (four-ten) batches. The protected aldehyde (1 mmol) was solved in 1.7 mL tetrahydrofuran and added dropwise to the stirred solution. Conversion was monitored using NMR. The reaction was quenched with 1 mol/L HCl. After phase separation the product was extracted from the aqueous phase three times with ethyl acetate. The combined organic layers were dried with $MgSO_4$ and filtered. The crude product after removal of the solvent was either

directly used for the reduction or purified by column chromatography when necessary.[5]

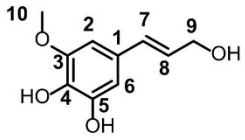
Reduction using DiBAl-H

DiBAl-H solution (5.5 eq., 1 M in dichloromethane) was diluted with 8.1 mL tetrahydrofuran. The solution was stirred and cooled to -20 °C. The ester (3 mmol) was solved in 2.3 mL tetrahydrofuran and added dropwise over 30 min. The reaction was stirred for 1 h at -20 °C and monitored using TLC. After complete conversion the excess DiBAl-H was converted using ethyl acetate. The resulting yellow solid was treated with 2 mol/L HCl. The product was extracted from the aqueous layer with diethylether (three times). The combined organic layers were washed with saturated NaCl-solution, dried with $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude product was purified using column chromatography.[2]

Reduction using $LiAlH_4$

$LiAlH_4$ (4 eq.) was added to 10.4 mL diethyl ether and stirred at -30 °C. The (*E*)-5-(3-ethoxy-3-oxoprop-1-en-1-yl)-3-methoxy-1,2-phenylene bis(2,2-dimethyl-propanoate) (1 mmol) in 7.8 mL diethyl ether was added dropwise over 30 min to the solution. The reaction was stirred at -20 °C for 2 h, while being monitored using TLC. After complete consumption of the ester remaining hydride was quenched with ethyl acetate at 0 °C. 2 mol/L HCl was added until the yellow residue was dissolved. The product 14 was extracted from the aqueous phase with diethyl ether (three times). The combined organic layers were washed with brine, dried with $MgSO_4$ and the solvent removed under reduced pressure. The product was immediately purified using column chromatography (*n*-pentane:ethyl acetate 60:40). The pure (*E*)**-3-(3,**4-**dihydr**oxy**-5-methoxy**)phenyl)**-**prop-2-en-1-ol (14) was stored under argon at -20 °C.

(*E*)-3-(3,4-Dihydroxy-5-methoxy)phenyl)-prop-2-en-1-ol (5-hydroxyconi-feryl alcohol) (14)

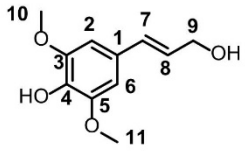


Yield: 33%

^{1}H-NMR ($CDCl_3$, 600 MHz) δ [ppm]: 3.84 (s, 3 H, 10-H); 4.21 (d, $^3J_{9,8}$ = 4.8 Hz, 2 H, 9-H); 6.21 (dt, $^3J_{8,7}$ = 15.7 Hz, $^3J_{8,9}$ = 5.2 Hz, 1 H, 8-H); 6.45 (d, $^3J_{7,8}$ = 15.9 Hz, 1 H, 7-H); 6.60 (d, $^4J_{2,6}$ = 2.3 Hz, 1 H, 2-H); 6.62 (d, $^4J_{6,2}$ = 2.3 Hz, 1 H, 6-H)

^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 31.8 (s, C-9); 55.5 (s, C-10); 101.6 (s, C-6); 107.1 (s, C-2); 127.4 (s, C-8); 129.8 (s, C-7); 133.6 (s, C-1); 145.4 (s, C-3); 147.9 (s, C-4 or C-5); 148.1 (s, C-5 or C-4)

(*E*)-3-(4-Hydroxy-3,5-dimethoxy)phenyl)-prop-2-en-1-ol (sinapyl alcohol) (15)

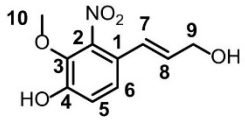


Yield: 71%

^{1}H-NMR ($CDCl_3$, 600 MHz) δ [ppm]: 3.90 (s, 6 H, 10-H and 11-H); 4.29-4.32 (m, 2 H, 9-H); 5.56 (s, 1 H, 4-O*H*); 6.24 (dt, $^{3}J_{8,7}$ = 15.8 Hz, $^{3}J_{8,9}$ = 5.9 Hz, 1 H, 8-H); 6.52 (dt, $^{3}J_{7,8}$ = 15.9 Hz, $^{4}J_{8,2/6}$ = 1.5 Hz, 1 H, 7-H); 6.63 (s, 2 H, 2-H and 6-H)

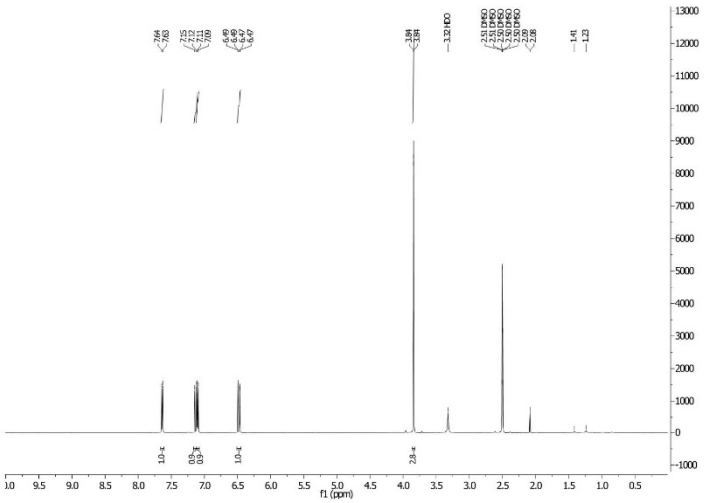
^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 56.4 (s, C-10 and C-11); 63.9 (s, C-9); 103.5 (s, C-2 and C-6); 126.7 (s, C-1); 128.4 (s, C-8); 131.6 (s, C-7); 134.9 (s, C-4); 147.3 (s, C-3 and C-5)

(*E*)-3-(4-Hydroxy-3-methoxy-2-nitro)phenyl)-prop-2-en-1-ol (2-nitroconiferyl alcohol) (23)

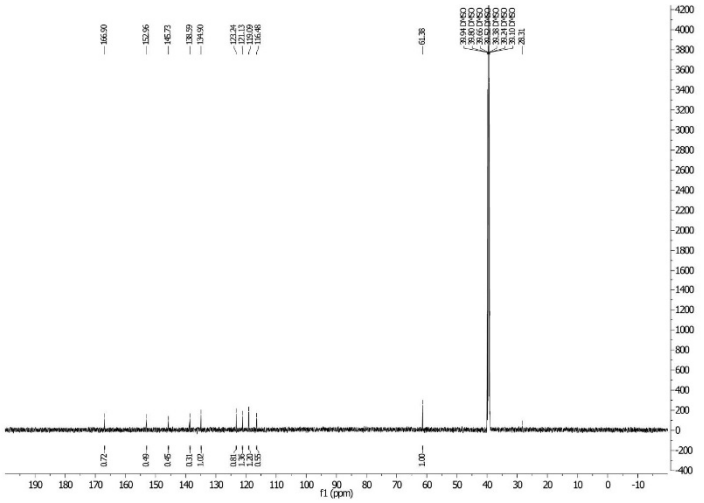


Yield: 39%

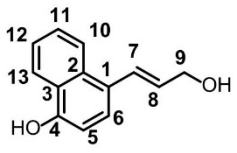
^{1}H-NMR (DMSO-d_6, 600 MHz) δ [ppm]: 3.81 (s, 3 H, 10-H); 4.06-4.09 (m, 2 H, 9-H); 4.94 (t, $^{3}J_{9\text{-OH},8}$ = 5.4 Hz, 1 H, 9-O*H*); 6.20 (dt, $^{3}J_{7,8}$ = 15.7 Hz, $^{4}J_{7,9}$ = 2.0 Hz, 1 H, 7-H); 6.35 (dt, $^{3}J_{8,7}$ = 15.7 Hz, $^{3}J_{8,9}$ = 4.5 Hz, 1 H, 8-H); 7.03 (d, $^{3}J_{6,5}$ = 8.7 Hz, 1 H, 6-H); 7.33 (d, $^{3}J_{5,6}$ = 8.7 Hz, 1 H, 5-H)



^{13}C-NMR (DMSO-d_6, 151 MHz) δ [ppm]: 61.0 (s, C-10); 61.2 (s, C-7); 118.9 (s, C-6); 119.2 (s, C-9); 119.6 (s, C-1); 121.4 (s, C-5); 134.0 (s, C-8); 138.2 (s, C-3); 144.6 (s, C-2); 149.9 (s, C-4)

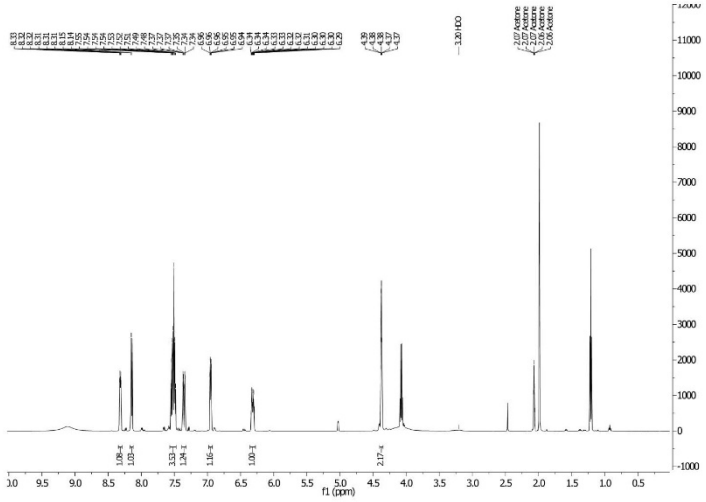


(*E*)-4-(3-Hydroxyprop-1-en-1-yl)naphthalen-1-ol (23)

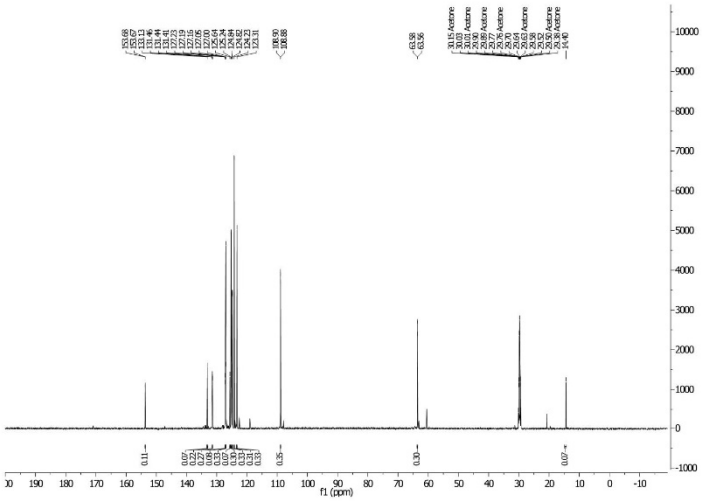


Yield: 52%

^{1}H-NMR (DMSO-*d*$_6$, 600 MHz) δ [ppm]: 4.38 (d, $^{3}J_{9,8}$ = 6.9 Hz, 2 H, 9-H); 6.32 (dt, $^{3}J_{8,7}$ = 16.1 Hz, $^{3}J_{8,9}$ = 6.6 Hz, 1 H, 8-H); 6.95 (d, $^{3}J_{13,12}$ = 7.8 Hz, 1 H, 13-H); 7.36 (d, $^{3}J_{7,8}$ = 16.1 Hz, 1 H, 7-H); 7.47-7.56 (m, 3 H, 10-H, 11-H and 12-H); 8.15 (d, $^{3}J_{5,6}$ = 8.4 Hz, 1 H, 5-H); 8.22-8.34 (m, 1 H, 6-H)



^{13}C-NMR (DMSO-*d*$_6$, 151 MHz) δ [ppm]: 63.6 (s, C-9); 108.9 (s, C-13); 123.3 (s, C-6); 124.2 (s, C-5); 124.8 (s, C-10 or C-11 or C-12); 125.2 (s, C-10 or C-11 or C-12); 125.6 (s, C-2); 127.0 (s, C-10 or C-11 or C-12); 127.0 (s, C-3); 127.2 (s, C-7); 131.4 (s, C-8); 133.1 (s, C-1); 153.7 (s, C-4)



Synthesis of (*E*)-3-(4-hydroxy-3-methoxy-2-nitrophenyl)acrylic acid

2-Nitrosylation

4-formyl-2-methoxyphenyl acetate (15 mmol) was added slowly under stirring to 0.4 mL of concentrated nitric acid (>90%) at -20 °C. The resulting solution was added to 8 g of ice. After filtration the resulting white solid was washed with water and then solved in 5% (w/v) NaOH-solution. The solution was acidified with 4 M HCl, resulting in precipitation of a white solid. After filtration this solid was washed with water and purified by column chromatography.[6]

HWE reaction

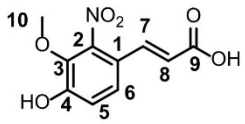
The triethyl phosphonoacetate (1.7 eq.) was solved in 1.6 mL tetrahydrofuran and the solution cooled to 0 °C. The NaH (1.7 eq., 60% suspension in mineral oil) was added in multiple (four-ten) batches. The 4-hydroxy-3-methoxy-2-nitro benzaldehyde (1 mmol) was solved in 1.7 mL tetrahydrofuran and added dropwise to the stirred solution. Conversion was monitored using NMR. The reaction was quenched with 1 mol/L HCl. After phase separation the product was extracted from the aqueous phase three times with ethyl acetate. The

combined organic layers were dried with $MgSO_4$ and filtered. The crude product after removal of the solvent was either directly used for the reduction or purified by column chromatography when necessary.[5]

Ester cleavage

The 3 ethyl (*E*)-3-(4-hydroxy-3-methoxy-2-nitrophenyl)acrylate (13 mmol) was solved in 1.5 mL ethanol. The solution was added dropwise to 20 mL 2.5 mol/L NaOH-solution. The reaction was stirred for 20 min at 24 °C and monitored with TLC. After complete conversion the reaction mixture was diluted with water, cooled to 0 °C and acidified with cold HCl. 2-Nitroferulic acid **(**(*E*)-3-(4-hydroxy-3-methoxy-2-nitrophenyl)acrylic acid**)** (20) was collected as a white precipitate after filtration.

(*E*)-3-(4-Hydroxy-3-methoxy-2-nitrophenyl)acrylic acid (2-nitroferulic acid) (**20**)



Yield: 38%

^{1}H-NMR (DMSO-d_6, 600 MHz) δ [ppm]: 3.84 (s, 3 H, 10-H); 6.47 (dd, $^{3}J_{7,8}$ = 15.7 Hz, $^{4}J_{7,6}$ = 1.8 Hz, 1 H, 7-H); 7.09 (dd, $^{3}J_{5,6}$ = 8.8 Hz, $^{4}J_{5,4\text{-}OH}$ = 1.7 Hz, 1 H, 5-H); 7.13 (d, $^{3}J_{8,7}$ = 15.7 Hz, 1 H, 8-H); 7.63 (dd, $^{3}J_{6,5}$ = 8.8 Hz, $^{4}J_{6,7}$ = 1.8 Hz,, 1 H, 6-H)

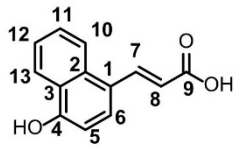
^{13}C-NMR (DMSO-d_6, 151 MHz) δ [ppm]: 61.4 (s, C-10); 116.5 (s, C-1); 119.1 (s, C-6); 121.1 (s, C-7); 123.2 (s, C-5); 134.9 (s, C-8); 138.6 (s, C-3); 145.7 (s, C-2); 153.0 (s, C-4); 166.9 (s, C-9)

Synthesis of Naphthalene Derivative

Demethylation

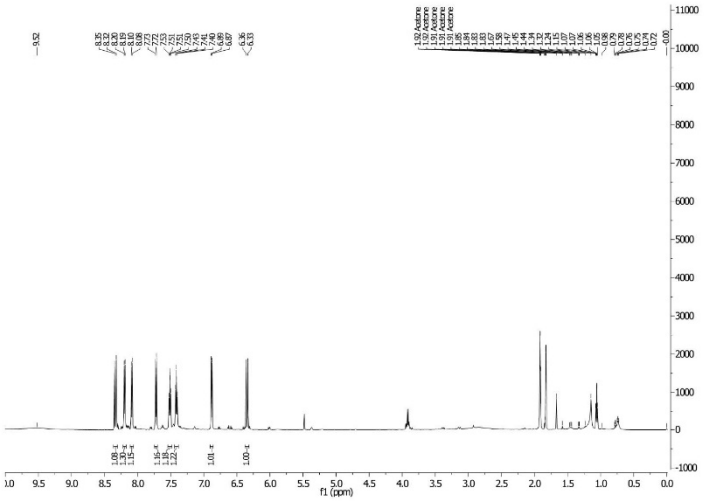
The (*E*)-3-(4-methoxynaphthalen-1-yl)acrylic acid (4.4 mmol) were solved in 11.5 mL dichloromethane. BBr_3-solution (3. 5 eq., 1 mol/L in dichloromethane) was added dropwise. The reaction was stirred for 20 h, while being monitored with TLC. After quenching with water the solution was diluted with ethyl acetate and water. The product was extracted from the aqueous layer with ethyl acetate. The combined organic layers were dried with $MgSO_4$, filtrated and the solvent removed under reduced pressure. The crude product 22 was purified with column chromatography.[7]

3-(4-Hydroxynaphthalen-1-yl)prop-2-enoic acid (22)

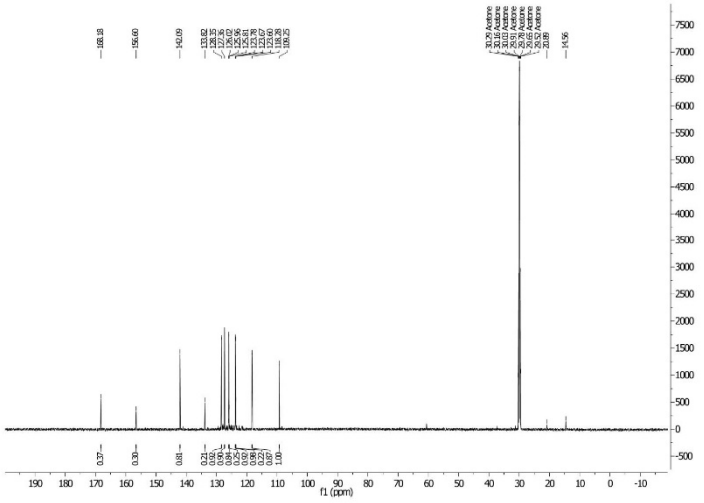


Yield: 35%

^{1}H-NMR (Acetone-d_6, 600 MHz) δ [ppm]: 6.35 (d, $^3J_{7,8}$ = 15.7 Hz, 1 H, 7-H); 6.88 (d, $^3J_{6,5}$ = 7.6 Hz, 1 H, 6-H); 7.39-7.43 (m, 1 H, 11-H); 7.49-7.53 (m, 1 H, 12-H); 7.72 (d, $^3J_{5,6}$ = 7.6 Hz, 1 H, 5-H); 8.09 (d, $^3J_{10,11}$ = 8.6 Hz, 1 H, 10-H); 8.20 (d, $^3J_{13,12}$ = 8.4 Hz, 1 H, 13-H); 8.34 (d, $^3J_{8,7}$ = 15.5 Hz, 1 H, 8-H)



^{13}C-NMR (Acetone-d_6, 151 MHz) δ [ppm]: 109.2 (s, C-5); 118.3 (s, C-7); 123.6 (s, C-1); 123.7 (s, C-10); 123.8 (s, C-13); 125.8 (s, C-2); 126.0 (s, C-12); 127.4 (s, C-6); 128.3 (s, C-11); 133.8 (s, C-3); 142.1 (s, C-8); 156.6 (s, C-4); 168.18 (s, C-9)



Absorption maxima and observed retention times of natural and non-natural cinnamic acid derivatives and their corresponding monolignols used for HPLC analyses. Benzoic acid always used as internal standard.

	Retention time [min]	Wavelength [nm]
Natural phenylpropanoids		
Cinnamic acid **(9)**	25.56	275
Cinnamyl alcohol **(6)**	19.38	250
p-Coumaric acid **(2)**	4.60	320
p-Coumaryl alcohol **(5)**	3.83	260
Caffeic acid **(10)**	2.75	325
Caffeoyl alcohol **(7)**	2.39	260
Ferulic acid **(11)**	5.90	325
Coniferyl alcohol **(8)**	4.70	260
Hydroxyferulic acid **(12)**	2.86	320
Hydroxyconiferyl alcohol **(14)**	3.88	250
Sinapic acid **(13)**	5.89	320
Sinapyl alcohol **(15)**	4.67	275
Non-natural phenylpropanoids		
3,4,5-Trimethoxycinnamic acid **(16)**	24.26	320
3,4,5-Trimethoxycinnamyl alcohol **(17)**	15.00	260
5-Bromoferulic acid **(18)**	23.66	325
5-Bromoconiferyl alcohol **(19)**	18.26	260
3-Nitroferulic acid **(20)**	17.30	275
3-Nitroconiferyl alcohol **(21)**	13.34	250
'Bicyclic' *p*-coumaric acid **22**	26.91	260
'Bicyclic' *p*-coumaryl alcohol **23**	28.86	320
Benzoic acid (internal standard)	9.14	230

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4.2 Supplementary material "Engineering the substrate specificity of a caffeic acid *O*-methyltransferase from *Medicago sativa*"

Supplementary Material

Engineering the substrate specificity of a caffeic acid *O*-methyltransferase from *Medicago sativa*

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Supplementary Material

Gene sequence, protein sequence and primer sequences

S I Gene sequence and protein sequence of caffeic acid *O*-methyltransferase (COMT) derived from *Medicago sativa*. Red colour indicates mutation sites.

Gene sequence	ATGGGTAGCACCGGTGAAACCCAGATTACCCCGACCCATATTAGTGA TGAAGAAGCAAACCTGTTTGCAATGCAGCTGGCAAGCGCAAGCGTT CTGCCGATGATTCTGAAAAGCGCACTGGAACTGGATCTGCTGGAAAT TATTGCAAAAGCAGGTCCGGGTGCACAGATTAGCCCGATTGAAATTG CAAGCCAGCTGCCGACCACCAATCCGGATGCACCGGTTATGCTGGA TCGTATGCTGCGTCTGCTGGCATGTTATATCATTCTGACCTGTAGCG TTCGTACCCAGCAGGATGGTAAAGTTCAGCGTCTGTATGGTCTGGCA ACCGTTGCAAAATATCTGGTGAAAAATGAAGATGGCGTTAGCATTAG CGCACTGAATCTGATGAATCAGGATAAAGTT**CTG**ATGGAAAGCTGGT ATCATCTGAAAGATGCAGTTCTGGATGGTGGTATTCCGTTTAACAAA GCATATGGTATGACC**GCC**TTTGAATATCATGGCACCGATCCGCGT**TT** **T**AACAAAGTGTTTAACAAAGGCATGAGCGATCATAGCACCATTACCA TGAAAAAAATCCTGGAAACCTATACCGGTTTTGAAGGTCTGAAAAGC CTGGTTGATGTTGGTGGTGGCACCGGTGCAGTGATTAATACCATTGT TAGCAAATACCCGACCATTAAAGGCATCAATTTTGATCTGCCGCATG TGATTGAAGATGCACCGAGCTATCCGGGTGTTGAACATGTGGGTGG TGATATGTTTGTTAGCATTCCGAAAGCAGATGCCGTGTTTATGAAATG GATTTGCCATGATTGGTCCGATGAACACTGTCTGAAATTTCTGAAAAA CTGCTATGAAGCCCTGCCGGATAATGGTAAAGTTATTGTTGCAGAAT GTATTCTGCCGGTTGCACCGGATAGCAGCCTGGCAACCAAAGGTGT TGTTCATATTGATGTTATTATGCTGGCACATAATCCGGGTGGTAAAGA ACGTACCCAGAAAGAATTTGAAGATCTGGCAAAAGGTGCAGGTTTTC AGGGTTTTAAAGTTCATTGCAATGCCTTTAATACCTATATTATGGAATT TCTGAAAAAAGTTTAA
Protein sequence	MGSTGETQITPTHISDEEANLFAMQLASASVLPMILKSALELDLLEIIAKA GPGAQISPIEIASQLPTTNPDAPVMLDRMLRLLACYIILTCSVRTQQDGKV QRLYGLATVAKYLVKNEDGVSISALNLMNQDKV**L**MESWYHLKDAVLDG GIPFNKAYGMT**A**FEYHGTDPR**F**NKVFNKGMSDHSTITMKKILETYTGFE GLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVIEDAPSYPGVEHVG GDMFVSIPKADAVFMKWICHDWSDEHCLKFLKNCYEALPDNGKVIVAE CILPVAPDSSLATKGVVHIDVIMLAHNPGGKERTQKEFEDLAKGAGFQG FKVHCNAFNTYIMEFLKKV

Supplementary Material

S II Primer used in this study. Nucleotides marked in red indicate mutation sites, the ribosome binding site (RBS) is marked in blue, stop codons are orange and restriction sites are underlined.

Name	Sequence (5' → 3')	Application
5MsEcComt_s	CTCAAGCTTAAGGAGGTAAGTAAT GGGTAGCACCGGTGAAACCCAG	*Hind*III, RBS
5MsEcComt_as2	ATTCTCGAGTTAAACTTTTTTCAGAA ATTCCATAATATAGGTATTAAAGGCA TTGCAATGAAC	*Xho*I, Stop
MsEcComtV135I_s	CTGATGAATCAGGATAAAATTCTGATG GAAAGCTGG	Substitution
MsEcComtV135I_as	CCAGCTTTCCATCAGAATTTTATCCTGA TTCATCAG	Substitution
MsEcComtA162T_s	CAAAGCATATGGTATGACCACCTTTGAA TATCATGGCAC	Substitution
MsEcComtA162T_as	GTGCCATGATATTCAAAGGTGGTCATAC CATATGCTTTG	Substitution
deg-L136-s	CTGATGAATCAGGATAAAGTTNNKATGG AAAGCTGGTATCATCTGAAAG	Library construction
deg-A162F172-as	CTCATGCCTTTGTTAAACACTTTGTTMNN ACGCGGATCGGTGCCATGATATTCAAA MNNGGTCATACCATATGCTTTGTTAAAC	Library construction
MsEcComtSS135_s	GCGCACTGAATCTGATGAATCAGGATAAA NNKCTGATGGAAAGCTGGT	Sublibrary construction
MsEcComtSS135_as	ACCAGCTTTCCATCAGMNNTTTATCCTGA TTCATCAGATTCAGTGCGC	Sublibrary construction
MsEcComtA09SS135_s	GCGCACTGAATCTGATGAATCAGGATAAA NNKCGGATGGAAAGCTGGT	Sublibrary construction
MsEcComtA09SS135_as	ACCAGCTTTCCATCCGMNNTTTATCCTGA TTCATCAGATTCAGTGCGC	Sublibrary construction
seq_pAL_s	ATATGGCTAGCATGACTGGTGGACAG	Sequencing
seq_C3H_as	CAGCAGCCAACTCAGCTTC	Sequencing

Supplementary Material

Quantification of phenylpropanoids

S III Used absorption maxima and observed retention times of hydroxycinnamic acid derivatives during HPLC quantification. Benzoic acid was used as internal standard.

Compound	Retention time [min]	Wave length [nm]
Caffeic acid	0.99	320
Ferulic acid	2.21	320
5-Hydroxyferulic acid	1.03	320
Sinapic acid	2.11	320
Benzoic acid	3.43	230

4.3 Authors' Contributions

Chapter 2.1:

Production of plant-derived polyphenols in microorganisms: current state and perspectives

Milke, L., Aschenbrenner, J., Marienhagen, J., and Kallscheuer, N. (2018). Production of plant-derived polyphenols in microorganisms: current state and perspectives. Applied Microbiology and Biotechnology, 102(4), 1575–1585. https://doi.org/10.1007/s00253-018-8747-5

J. Aschenbrenner, L. Milke and N. Kallscheuer performed literature search and collected and compiled the material. J. Aschenbrenner, L. Milke, N. Kallscheuer and J. Marienhagen discussed the structure of the manuscript. J. Aschenbrenner, L. Milke, N. Kallscheuer and J. Marienhagen revised the manuscript.

Chapter 2.2:

Microbial production of natural and non-natural monolignols with *Escherichia coli*

Aschenbrenner, J., Marx, P., Pietruszka, J. and Marienhagen, J. (2018). Microbial production of natural and non-natural monolignols with *Escherichia coli*. ChemBioChem, [Epub ahead of print]. https://doi.org/10.1002/cbic.201800673

J. Aschenbrenner planned and performed the experiments. P. Marx synthesised non-natural substrates, the reference material and performed NMR measurements. J. Marienhagen and J. Pietruszka wrote the manuscript based on the first draft written by J. Aschenbrenner. P. Marx wrote the supporting information.

Chapter 2.3:

Engineering the substrate specificity of a caffeic acid *O-methyltransferase* from *Medicago sativa*

Aschenbrenner, J., Jansen, S., and Marienhagen, J.

As final studies have to be performed, the manuscript could not be finalized until this thesis was submitted. Thus, this chapter comprises a manuscript draft focusing on the obtained results. J. Aschenbrenner planned the project, the experiments and implemented the screening system. S. Jansen constructed the plasmids, performed site-saturation mutagenesis and cultivations, which was supervised by J. Aschenbrenner. J. Aschenbrenner wrote the manuscript.

4.4 Other activities

Poster presentations

J. Aschenbrenner, P.v.Summeren-Wesenhagen, R. Voges, S. Sokolowsky, S. Noack J. Marienhagen. Combinatorial optimization of synthetic operons for the microbial production of *p*-coumaryl alcohol with *Escherichia coli*, 2015, EMBO | EMBL Symposium: New Approaches and Concepts in Microbiology, Heidelberg.

J. Aschenbrenner, P. Marx, J. Pietruszka and J. Marienhagen. Combinatorial biosynthesis of natural and non-natural plant-derived phenols in microorganisms, 2017, 7th Congress of European Microbiologists (FEMS).

Oral presentations

J. Aschenbrenner, P.v.Summeren-Wesenhagen, R. Voges, S. Sokolowsky, S. Noack J. Marienhagen. Combinatorial optimization of synthetic operons for the microbial production of *p*-coumaryl alcohol with *Escherichia coli*, 2016, 17th European Congress on Biotechnology (ECB).

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Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

_____ _____

Ort, Datum Jennifer Aschenbrenner