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

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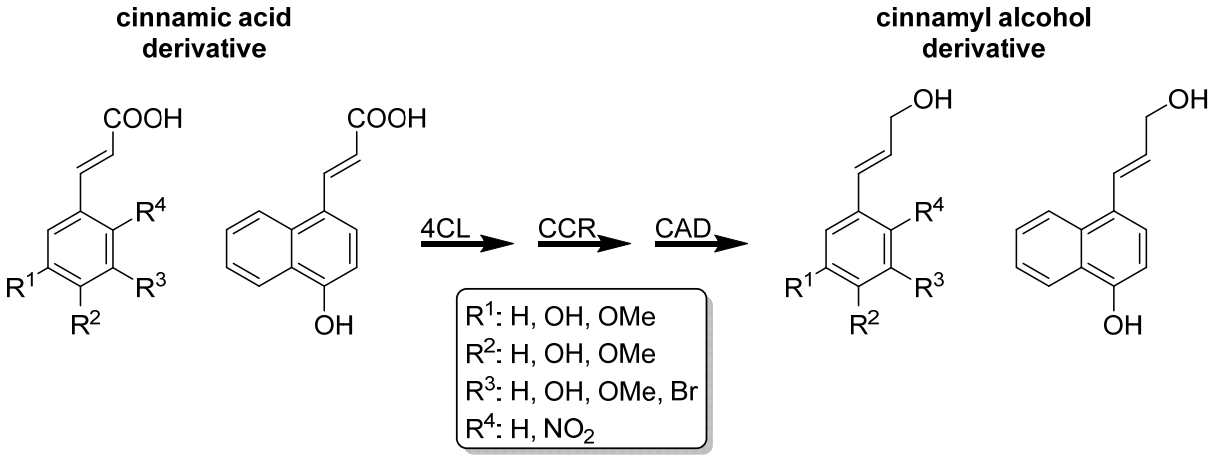

One for all. The promiscuity of three enzymes constituting a synthetic phenylpropanoid pathway in *Escherichia coli* was exploited for the stepwise reduction of six naturally occuring as well as four non-natural phenylpropenoic acids to their corresponding monolignols.

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

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).

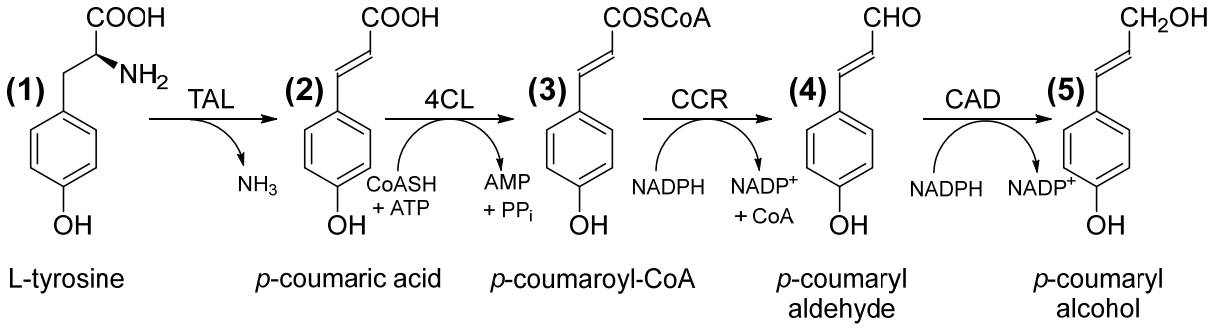

Figure 1. Biosynthetic pathway for *p*-coumaryl alcohol synthesis from L-tyrosine. TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase.

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

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

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

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

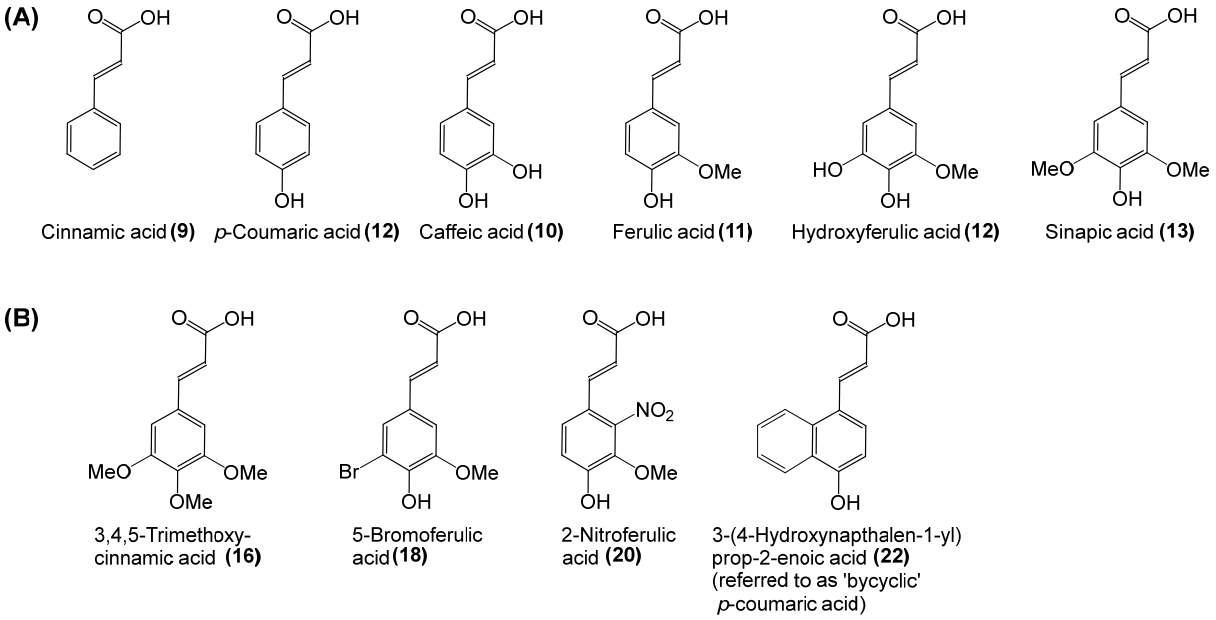

Figure 2. (A) Naturally occurring and **(B)** non-natural cinnamic acid derivatives used in this study.

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

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*-

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

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

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

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]

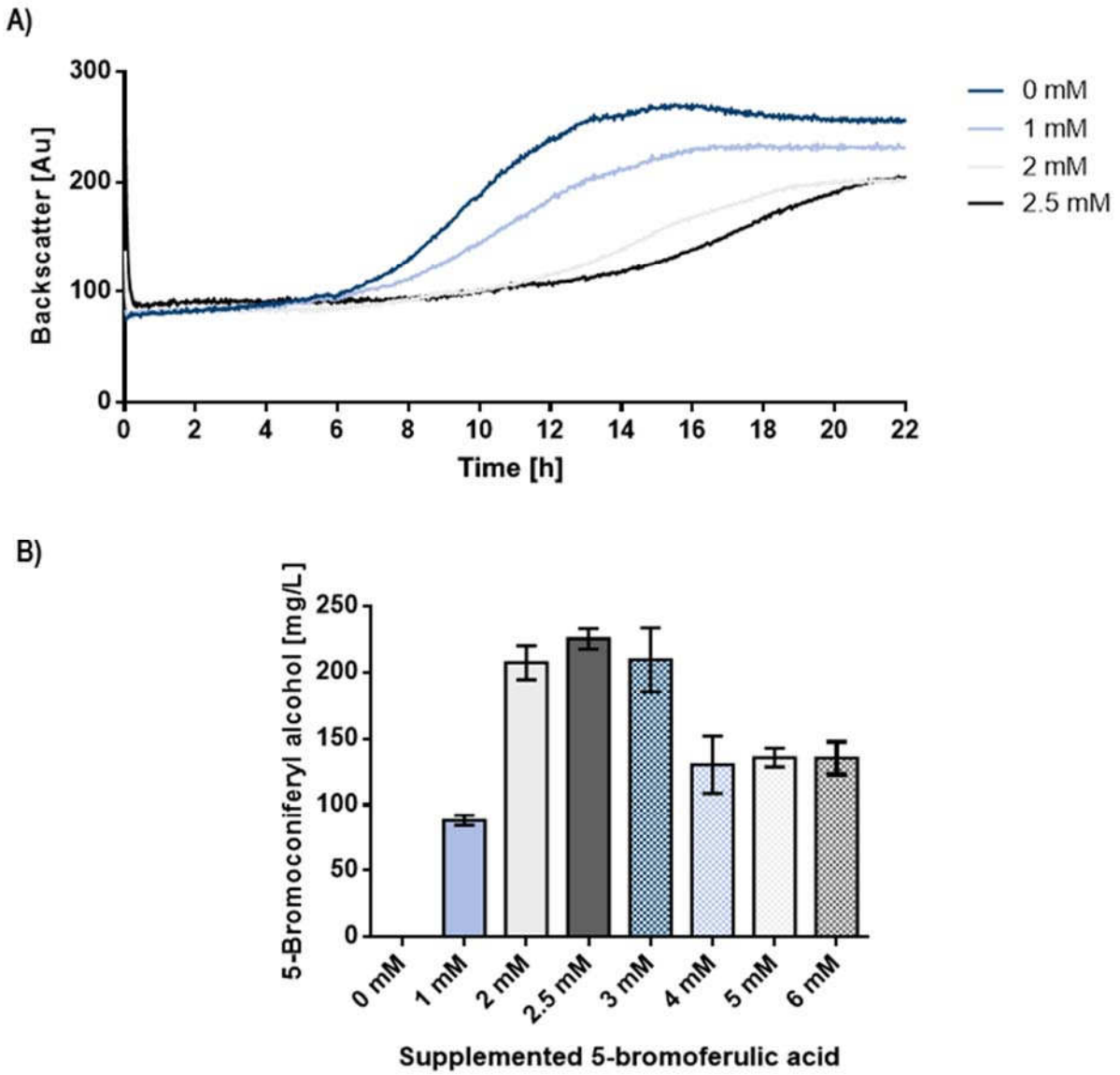

Figure 3. Impact of different 5-bromoferulic acid (**18**) concentrations on cell growth and 5-bromoconiferyl alcohol (**19**) production. A) Growth of the engineered *E. coli* strain in the presence of 5-bromoferulic acid (**18**) concentrations ranging from 0 mM to 2,5 mM B) Obtained 5-bromoconiferyl 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.

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.

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

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 (**Fehler! Verweisquelle konnte nicht gefunden werden.**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

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

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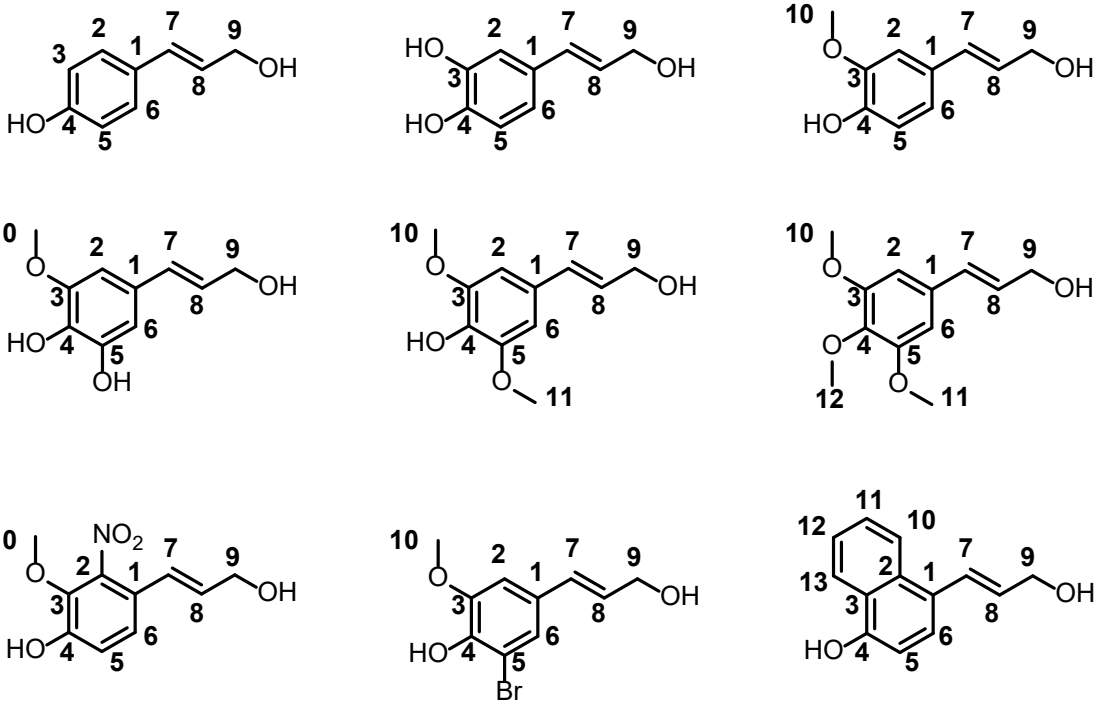
List of contents

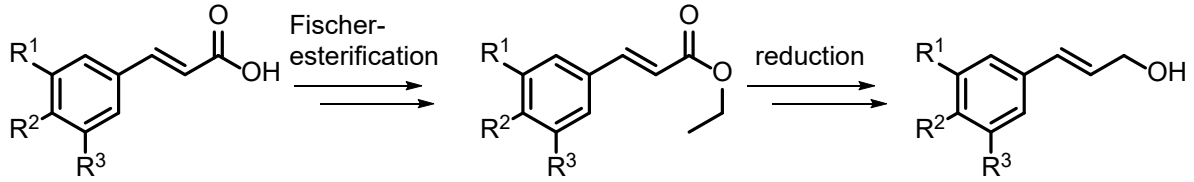
General syntheses

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



R^1 = H, OH, OMe
R^2 = OH, OMe
R^3 = H, OMe, Br

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]

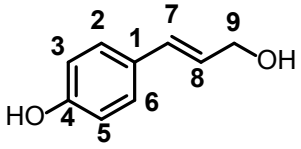
Ester reduction using DiBAl-H

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)

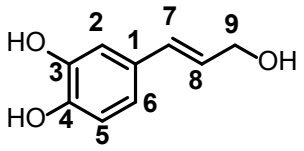


Yield: 35%

^{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)

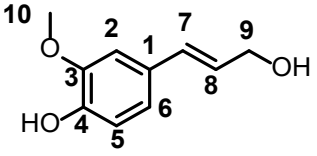


Yield: 43%

^{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)



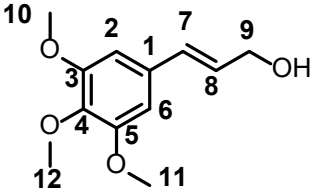
Yield: 90%

^{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)

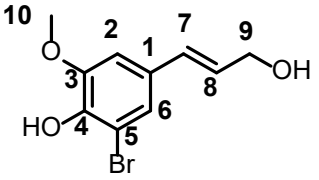


Yield: 46%

^{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)

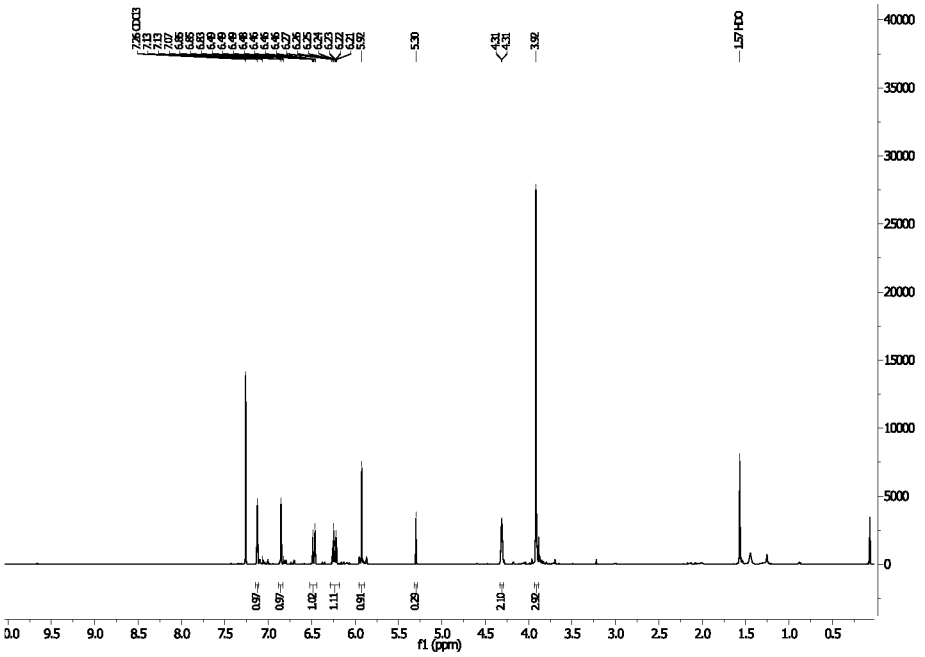
(*E*)-3-(5-Bromo-4-hydroxy-3-methoxy)phenyl)-prop-2-en-1-ol (5-bromo-coniferyl alcohol) (19)



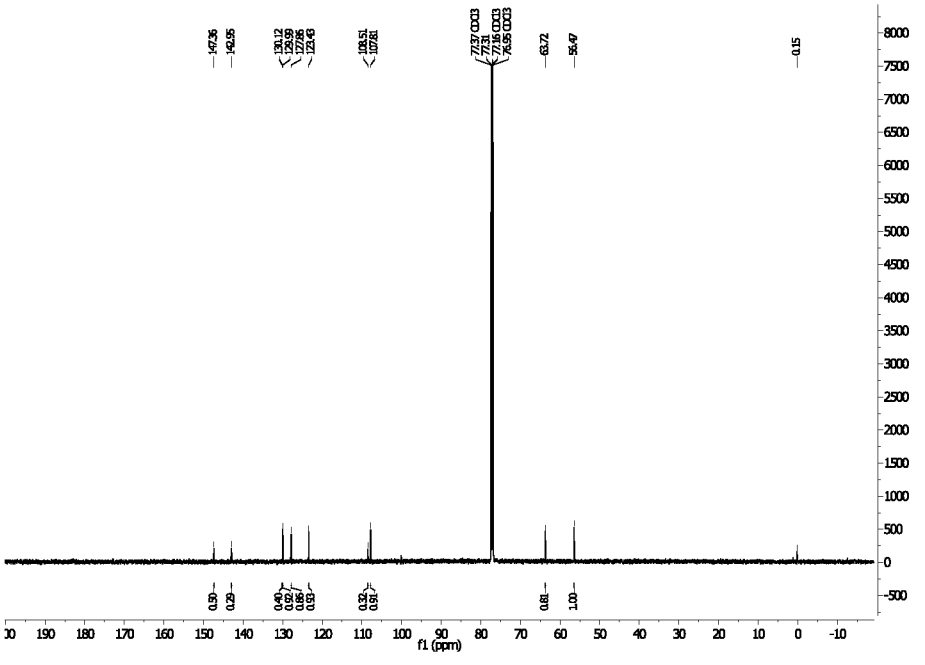
Yield: 80%

^{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

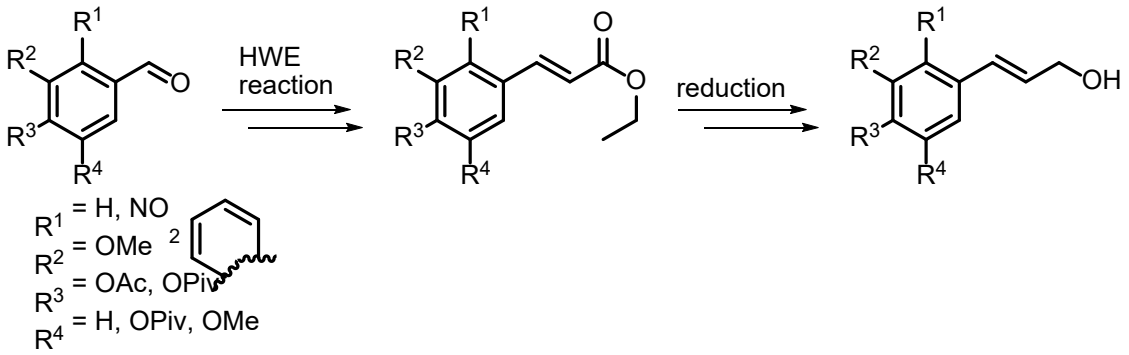
H, 8-H); 6.47 (d, $^{3}J_{7,8}$ = 15.8 Hz, 1 H, 7-H); 6.85 (d, $^{4}J_{6,2}$= 1.8 Hz, 1 H, 6-H); 7.13 (d, $^{4}J_{2,6}$= 1.8 Hz, 1 H, 2-H)



^{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]

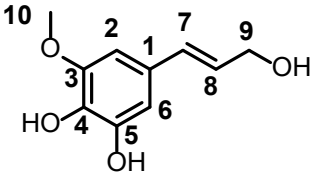
Reduction using DiBAl-H

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-hydroxyconiferyl alcohol) (14)

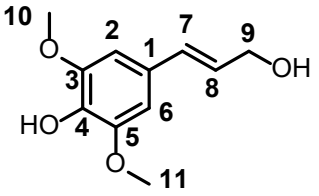


Yield: 33%

^{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)

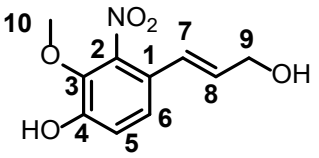


Yield: 71%

^{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)

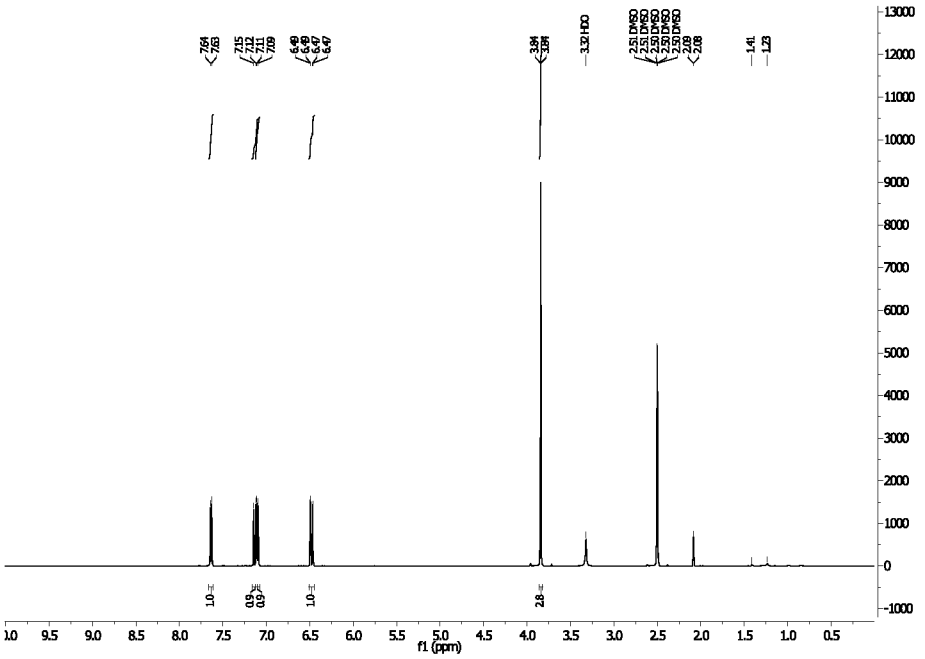
^{13}C-NMR ($CDCl_3$, 151 MHz) δ [ppm]: 56.4 (s, C-10 and C-11); 63.9 (s, C-9); 103.5 (s, C-2 and C-6); 126.7 (s, C-1); 128.4 (s, C-8); 131.6 (s, C-7); 134.9 (s, C-4); 147.3 (s, C-3 and C-5)

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

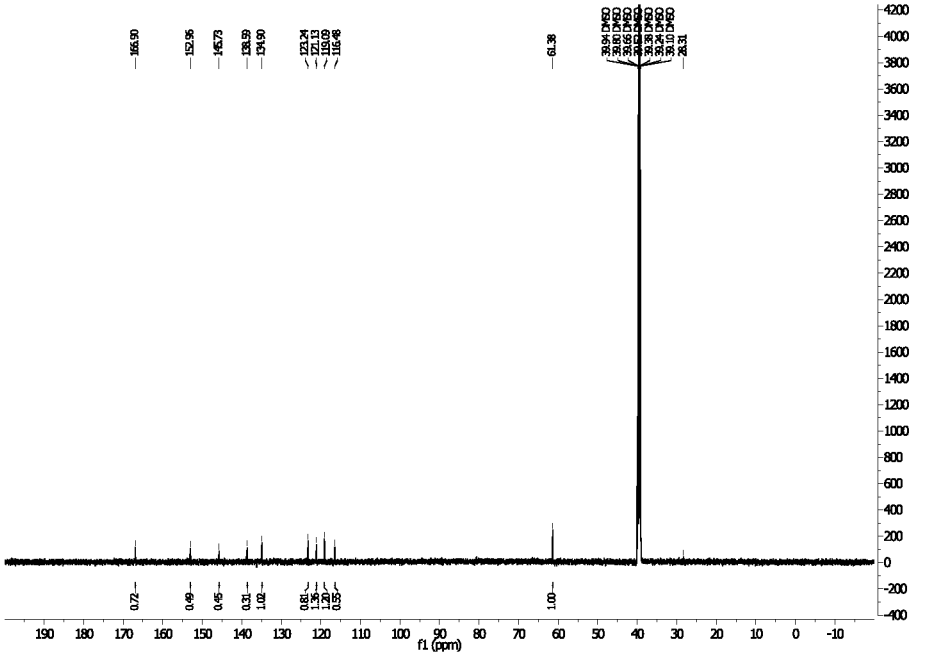


Yield: 39%

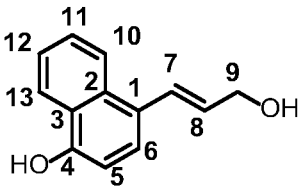
^{1}H-NMR (DMSO-d_6, 600 MHz) δ [ppm]: 3.81 (s, 3 H, 10-H); 4.06-4.09 (m, 2 H, 9-H); 4.94 (t, $^{3}J_{9\text{-OH},8}$ = 5.4 Hz, 1 H, 9-O*H*); 6.20 (dt, $^{3}J_{7,8}$ = 15.7 Hz, $^{4}J_{7,9}$ = 2.0 Hz, 1 H, 7-H); 6.35 (dt, $^{3}J_{8,7}$ = 15.7 Hz, $^{3}J_{8,9}$ = 4.5 Hz, 1 H, 8-H); 7.03 (d, $^{3}J_{6,5}$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

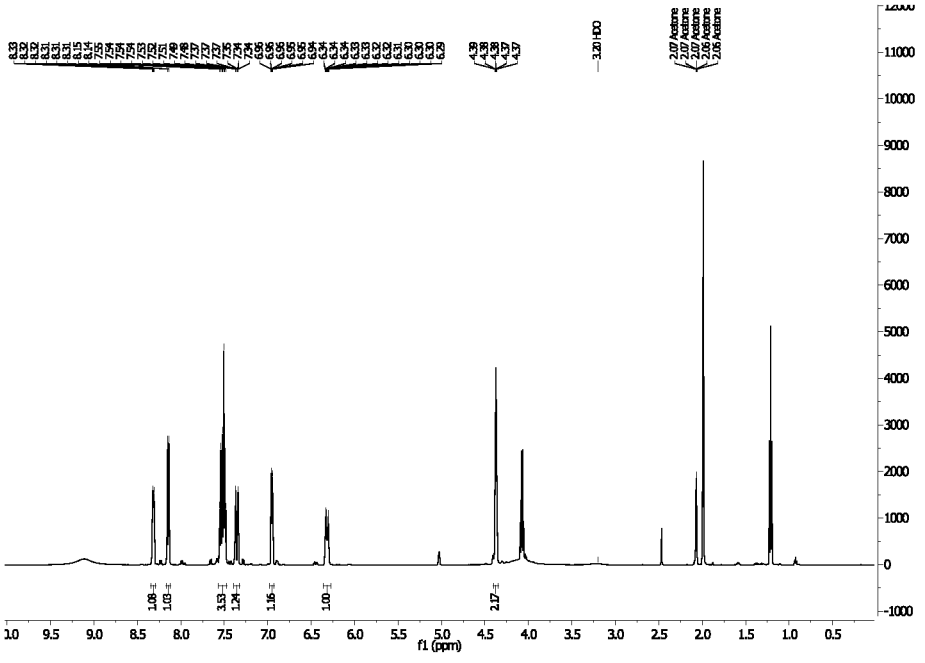


(*E*)-4-(3-Hydroxyprop-1-en-1-yl)naphthalen-1-ol (23)

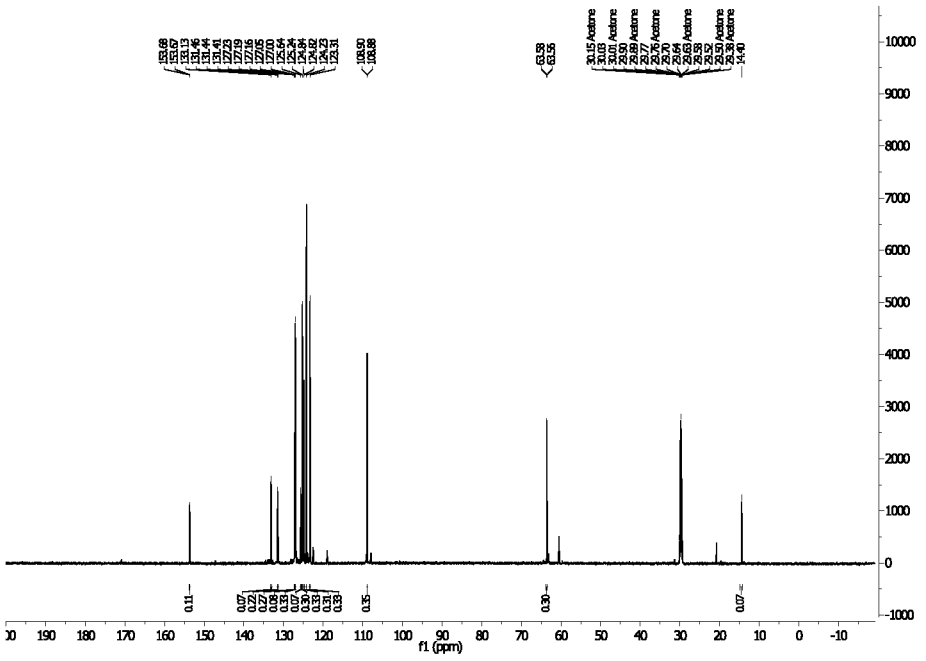


Yield: 52%

^{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

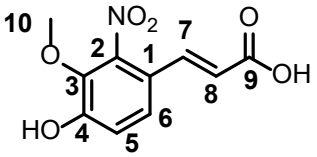
The triethyl phosphonoacetate (1.7 eq.) was solved in 1.6 mL tetrahydrofuran and the solution cooled to 0 °C. The NaH (1.7 eq., 60% suspension in mineral oil) was added in multiple (four-ten) batches. The 4-hydroxy-3-methoxy-2-nitro benzaldehyde (1 mmol) was solved in 1.7 mL tetrahydrofuran and added 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)

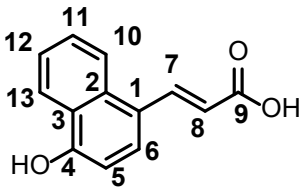
^{13}C-NMR (DMSO-d_6, 151 MHz) δ [ppm]: 61.4 (s, C-10); 116.5 (s, C-1); 119.1 (s, C-6); 121.1 (s, C-7); 123.2 (s, C-5); 134.9 (s, C-8); 138.6 (s, C-3); 145.7 (s, C-2); 153.0 (s, C-4); 166.9 (s, C-9)

Synthesis of Naphthalene Derivative

Demethylation

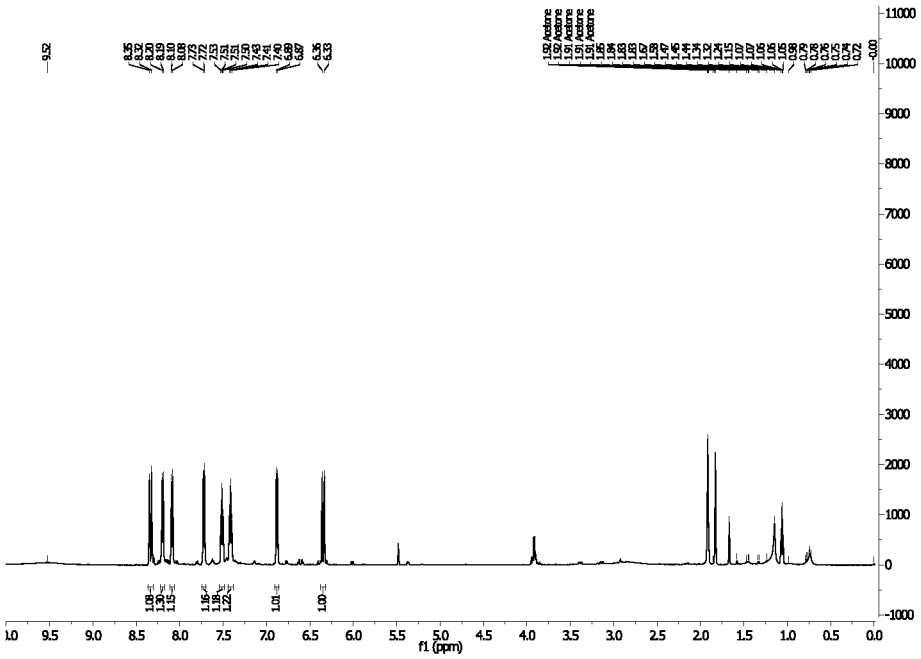
The (*E*)-3-(4-methoxynaphthalen-1-yl)acrylic acid (4.4 mmol) were solved in 11.5 mL dichloromethane. BBr_3-solution (3. 5 eq., 1 mol/L in dichloromethane) was added dropwise. The reaction was stirred for 20 h, while being monitored with TLC. After quenching with water the solution was diluted with ethyl 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)

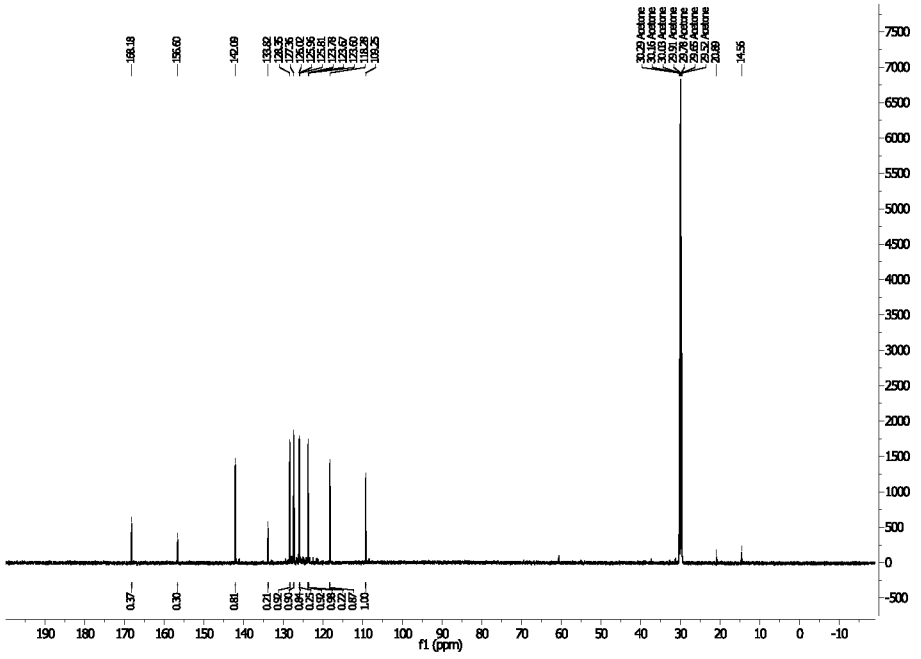


Yield: 35%

^{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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