Synthesis of (-)-deoxypodophyllotoxin and (-)-epipodophyllotoxin via a multi-enzyme cascade in *E. coli*

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Supplementary Information

1. Supplementary Material

omt1 (GenBank KT390155.1), native DNA-sequence

ATGGATACTAGGGCTGATGCTGAGATTAAAGCAATGGAGCTGATTGGTATTGGAGTACTTCCACTGGCAATGA
AGGCGATAATTGAGCTCAATGTGTTAGAGATCCTATCAAAAGCAGGACCAGATACCCAACTCACTGCTGCTCA
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ACTTTCTCCCAGATCAAGATGGAGTCTCACTAGCACCTATGGTTGTTACCATCCATGACAAAGTGCTGCTTCA
AAGTTGGCATTATCTTAAGGACTCTGTTTTAAAACAAGGCTCTTTGCCATTTACCGAGGCCTTTGGGATGTCA
CCCTTTGAGTATTCTGTCTCCGATACAAGGTTTAATAAGGTTTTTAATGCTGGCATGTTTGACCATTCTACTC
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AACTGGCGGATCGTTGAAGATGATTCTTTCTCAGTACCCCAATCTAAAGGGCATCAATTTTGATCTCCCACAT
GTGGTTGCCGACGCGCCTTCTTTTCCTGGTGTGAAGCACATTGGTGGTGATATGTTTGAGAGTGTTCCCTCTG
GTGATGCAATTTTCATGAAGTGGATACTTCATGACTGGGACGATGGACGTTGCCTGACTTTGCTGAAGAATTG
TTGGAATGCATTGCCAGAGCATGGAAAGGTGATAATAGTGGAGTGGATTCTACCATCAGATGCAGCGACTGAC
CCAACATCTCGCCGTGTCTTCACAGCTGATTTGATGATGTTGGCTTTCAGCGAAGGGGGTAAAGAGCGAACCT
TGGGTGACTACGGAGCACTTGCAAAGGAAGCTGGTTTTACCACTGTCAAAGATTTCCCTTGCGCAAATGGCAT
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omt3 (GenBank KT390157.1), native DNA-sequence

ATGGAAATGGCTCCAACAATGGATTTAGAGATAAGAAATGGAAATGGTTATGGTGATTCTGGAGAGGAGCTTC
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CAAATCCCCCAAGCAAAATCCGCGTGTCTGTATCGCCTGTTGCGAATACTAGTCCATTCTGGCTTCTTTGCCA
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***cyp71cu1* (GenBank KT390172.1), native DNA-sequence (*cyp71cu1nat_wt*)**

N-terminally truncated part of the DNA-sequence (*CYP71CU1Δ20nat*) is highlighted in bold and underlined

ATG**GAAACATTTCAGTGCCTCACACTTTTCCTTCTCTTCATCTCCACTGTTTTCATCCTCAAG**AGAAAATTCT
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TCCTATTTCTCGATCCTCTTGA

***cyp71cu1*, *E. coli* codon optimized DNA-sequence (c*yp71cu1oc_wt*)**

N-terminally truncated part of the DNA-sequence (*cyp71cu1Δ20oc*) is highlighted in bold and underlined

ATG**GAAACCTTCCAGTGCCTGACCCTGTTCCTGCTGTTCATCTCTACCGTTTTCATCCTGAAA**CGTAAATTCT
CTCACAAACCGAACCTGCCGCCGTCTCCGCCGAAACTGCCGATCCTGGGTAACTTCCACCAGCTGGGTACCCT
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GCGTGAAATGAAAAAAATGGCTGTTCTGAACCTGCTGTCTATCAAAAAAATCCAGTCTTTCCGTTCTGTTCGT
GAAGAACTGGCTTCTGACATGATCAAAGAAATCACCCGTCTGTCTAAAACCGGTGCTCCGGTTGACGTTACCA
ACATGCTGTACCACTTCTCTGAAGACCTGCTGTTCCGTTGCACCCTGGGTTTCAAACCGAAAGGTCAGCACAA
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CCTGGGTGGTATCGACCTGCCGGCTTCTGTTATGGAATGGGCTATGGCTGAACTGATGCGTAACCCGTCTAAA
ATGAAAATCGCTCAGGAAGAAGTTCGTAAAGTTGTTGGTAACAAAAACAAAGTTGACGAAGACGACGTTTACC
AGATGAACTTCCTGAAATCTGCTGTTAAAGAAACCCTGCGTCTGCACCCGCCGGCTCCGCTGCTGTTCGCTCG
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CACATCCAGCGTGACCCGAAACTGTGGGACAAAGCTGAAGAATTTATCCCGGAACGTTTCATGAACTCTGGTA
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CGGTGTTGCTGCTGTTGAATTTGCTGTTGCTAACCTGCTGTACTGGTTCGACTGGAAATTCGTTGGTGACACC
ACCCCGGAAACCCTGGACATGACCGAAGACTACTGCTTCGCTCTGTTCAAAAAAAAACCGCTGCACTTCATCC
CGATCTCTCGTTCTTCTTAA

***2-odd* (GenBank KT390173.12), native DNA-sequence**

ATGGGTTCTACAGCACCCCTAAGGCTTCCAGTTATAGATTTATCCATGAAGAACTTGAAGCCTGGAACAACTT
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TGTGTCTCCAGAGCTGCAGAAGGCAGTATGTAACAAAGGACACGAGCTGCTTAATCTTCCATTAGAAACCAAG
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GCAGAATAACAGATTTGGAAAAAGTTGAGAGGTTCACTAATCTTATGTGGCCCGAGGGGAATAAGGATTTCTG
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AACCAGGGAAGGATGGACGTTCACCTGCTCTTTCGTTGCATAAGGACAAGAGCATCTTGACCATAGTAAACCA
AAATGATGTCAAGGGATTGGAATTTGAAACCAAGGATGGAGAATGGATTTTACCTACAGCTGACAACCATATT
GTTCTTCTAGGAGACTGCTTCATGGCATGGAGCAATGGTAGATTACATAGTCCTCTTCACCGGGTCACGTTGG
TCGCGAACCAGGCGAGGTTATCTACATCATCGTTTTCGTTTCCAAAGGACATAATAGAGACCCCTGCAGAGCT
GGTGGATGAAGAGCATCCTTTGCTATTTAATCCCTTTGAGATAACGGAGTTGCTTGCTTACTGTTTCACAAAA
GAGGGTGCAAAGGCGGTGTGTGACCTCAAGCAATACAAGGCGTACACAGGTGCATGA

***cyp82d61*, *E. coli* codon optimized DNA-sequence (c*yp82d61oc_wt*)**

N-terminally truncated part of the DNA-sequence (*cyp82d61*Δ*23oc*) is highlighted in bold and underlined; for native DNA-sequence of *cyp82d61* gene see GenBank KC110995.1

ATG**GACTCTCTGCACTGCCTGGAAACCCTGCTGCTGGGTTTCTTCGTTCTGCTGCCGTGCTTCTTCTACTTC**G
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CCCAGAACATCGACTTCTGCCTGGTTGACATGAAACAGTGGCTGGCTGACCTGACCCTGAACATGTCTGTTAA
AATGGTTGTTGGTAAACGTTTCTTCGGTTCTGCTTCTGCTTCTGCTTGCGAAGAAACCGAATCTTCTAACTGC
CCGAAAACCCTGCGTAACATGTTCCGTCTGATGGGTTCTTTCGTTCTGTCTGACTACCTGCCGTACCTGCGTT
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CAAAGTTGACATGACCGAAACCCCGGGTATCGTTCACGCTAAAGCTACCCCGCTGGAAGTTCTGGTTGCTCCG
CGTATCTCTCCGAAATGCTTCGTTTAA

2. Supplementary Methods

Expressions of OMT3, OMT1 and 2-ODD

Expressions were evaluated in *E. coli* BL21 (DE3), C41(DE3) and C43 (DE3). Protein expression was induced at OD_{600} = 0.6 by the addition of 0.5 mM IPTG (additional supplementation with 0.1 mM $FeSO_4$ in the case of 2-ODD), followed by incubation at 25°C, 120 rpm for 20 h.

Expressions of wildtype and N-terminal truncated CYP71CU1 and CYP82D61 variants

Expressions were evaluated in *E. coli* C41(DE3). Protein expression was induced at OD_{600} = 0.6 by the addition of 0.5 mM IPTG, with additional supplementation with 0.5 mM 5-aminolevulinic acid (5-ALA) and 0.1 mM $FeSO_4$. Incubations were carried out at 120 rpm for 20 h or 48 h, with temperatures of 20°C, 25°C and 30°C. Cells were harvested by centrifugation (3,220 x g, 4°C, 20 min) and the cell pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.5, 100 µM PMSF. Cells were disrupted by sonication, the cell debris was sedimented by centrifugation (12,300 x g, 4°C ,30 min) and separated from the soluble protein fraction.

P450 concentrations within the soluble protein fractions were determined by recording CO difference spectra according to the method of Omura and Sato [1] to allow comparison between wildtype and truncated versions of CYP71CU1 and CYP82D61.

The results of the screening and optimized expression conditions are summarized in Tables S8, S9, and S10 within this appendix.

Table S1. Summary of generated *cyp71cu1 and cyp82d61* gene variants.

Name of variant	DNA-sequence	Inserted modification (second amino acid)	No. of N-terminal truncated amino acids
cyp71cu1Δ20nat	Native	Ala	1 - 20 (Δ20)
cyp71cu1Δ20oc	*E. coli* codon optimized	Ala	1 - 20 (Δ20)
cyp82d61Δ23oc	*E. coli* codon optimized	Ala	1 - 23 (Δ23)

Table S2. Summary of genes used for establishment of multi-enzyme cascade reactions.

Gene	Complete name or purpose	Organism	Reference
cyp719aΔ23oc	(-)-pluviatolide synthase (truncated, codon optimized)	*Sinopodophyllum hexandrum*	Decembrino *et al.*, 2020 [2]
atr2	NADPH-cytochrome P450 reductase 2 (ATR2)	*Arabidopsis thaliana*	Kranz-Finger *et al.*, 2018 [3]
omt3	(-)-pluviatolide-O-methyltransferase	*Sinopodophyllum hexandrum*	This work
cyp71cu1Δ20nat	(-)-5'-desmetoxy-yatein hydroxylase (truncated, native DNA-sequence)	*Sinopodophyllum hexandrum*	This work
omt1	(-)-5'-desmethyl-yatein O-methyltransferase	*Sinopodophyllum hexandrum*	This work
2-odd	(-)-deoxypodophyllotoxin synthase	*Sinopodophyllum hexandrum*	This work
cyp82d61Δ23oc	Putative hydroxylation (truncated, codon optimized)	*Sinopodophyllum hexandrum*	This work

Table S3. Summary of plasmids used within this study.

Plasmid	Features	Copy number	Reference
pETDuet_atr2_cyp719aΔ23oc	ColE1 ori, P_{T7}, *lacI,* Amp^R	~ 40	Decembrino *et al.,* 2020 [2]
pCDFDuet_omt3_cyp71cu1Δ20nat	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pCDFDuet_omt3	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pCDFDuet_cyp71cu1Δ20nat	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pCDFDuet_cyp71cu1Δ20oc	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pCDFDuet_cyp71cu1nat_wt	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pCDFDuet_cyp71cu1oc_wt	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pCOLADuet_2-odd_omt1	ColA ori, P_{T7}, *lac,* Kan^R	20 - 40	This work
pCDFDuet_omt1	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pCDFDuet_2-odd	CloDf13 ori, P_{T7}, *lac,* Sm^R	20 - 40	This work
pETDuet_atr2_cyp82d61Δ23oc	ColE1 ori, P_{T7}, *lacI,* Amp^R	~ 40	This work
pETDuet_cyp82d61oc_wt	ColE1 ori, P_{T7}, *lacI,* Amp^R	~ 40	This work
pETDuet_cyp82d61Δ23oc	ColE1 ori, P_{T7}, *lacI,* Amp^R	~ 40	This work

Table S4. Oligonucleotides used for generation of *cyp71cu1 and cyp82d61* gene variants.

Name of variant	Oligonucleotides 5' → 3'
cyp71cu1Δ20nat	**forward:** CAGAAAATTCTCTCATAAACCAAAC **reverse:** GCCATATGTATATCTCCTTCTTTATAC
cyp71cu1Δ20oc	**forward:** CCGTAAATTCTCTCACAAAC **reverse:** GCCATATGTATATCTCCTTCTTATAC
cyp82d61Δ23oc	**forward:** CGTTTGGAAAAAACCGAAC **reverse:** GCCATATGTATATCTCCTTCTTATAC

Table S5. LC/MS methods used within this study. Gradients were made of methanol (solvent A) and ddH_2O + 0.1% formic acid (solvent B). A flow rate of 0.5 mL/min was employed with methods 1, 2 and 3, whereas 0.8 mL/min was used in case of method 4.

	Time [min]	Solvent B [%]
Method 1	0.01	57
	8.00	28
	13.00	0
	14.01	57
	20.00	57

	Time [min]	Solvent B [%]
Method 2:	0.01	80
	5.00	65
	10.00	65
	20.00	38
	25.00	0
	26.01	80
	35.00	80

	Time [min]	Solvent B [%]
Method 3	0.01	65
	5.00	50
	8.00	45
	22.00	38
	25.00	0
	26.01	80
	35.00	80

	Time [min]	Solvent B [%]
Method 4	0.01	80
	2.00	60
	5.00	55
	25.00	52
	32.50	0
	35.01	80
	40.00	80

Qualitative analysis

Table S6. Authentic reference compounds used for LC/MS identification of reaction products. Commercially not available compounds were identified via their characteristic m/z fragments as described elsewhere [4].

Compound	Manufacturer or reference	Purity	Molecular weight	RT [min] (method 4)	Characteristic m/z fragments
(-)-matairesinol 1	Phytolab	≥99% (HPLC)	358.38	9.0	359 $[M+H]^+$ 341 $[M+H-H_2O]^+$
(-)-pluviatolide 2	*Decembrino et al. 2020* (isolated in-house) [2]	≥95% (HPLC)	360.61	15.0	357 $[M+H]^+$ 339 $[M+H-H_2O]^+$
(-)-yatein 5	BioBioPha	≥97% (HPLC)	400.40	20.6	401 $[M+H]^+$ 383 $[M+H-H_2O]^+$
(-)-deoxypodophyllotoxin 6	Toronto Research Chemical	≥98% (HPLC)	398.40	19.8	399 $[M+H]^+$ 421 $[M+Na]^+$
(-)-5'-desmethoxy-yatein 3	Commercially not available [4]	-	370.40	21.0	371 $[M+H^+]^+$ 353 $[M+H-H_2O]^+$
(-)-5'-desmethyl-yatein 4	Commercially not available [4]	-	386.40	14.8	387 $[M+H]^+$ 369 $[M+H-H_2O]^+$
(-)-epipodophyllotoxin 7	Commercially not available [4]	-	414.41	10.4	415 $[M+H]^+$ 437 $[M+Na]^+$
(+)-sesamin (internal standard; IS)	TCI	>98% (GC)	354.35	31.4	337 $[M+H-H_2O]^+$

Underlined m/z fragments correspond to those referred by Lau and Sattely [4].

Quantitative analysis

Table S7. For quantitative analysis, substrate conversion values were calculated by the product distribution resulting from the MS-peak areas of reaction intermediates/products and substrate. Estimation of the concentration of the final product (-)-deoxypodophyllotoxin **6** was done via internal calibration in the range of 10 - 300 µM utilizing 200 µM (+)-sesamin as IS.

Conversion [%] Normalized to IS	$= 1- (S_{sample} / IS_{sample}) / (S_{control} / IS_{control})$
Conversion [%]	$= \Sigma(P_{area}) / \Sigma(S_{area} + P_{area}) * 100$
Product distribution [%]	$= P_{area} / \Sigma(S_{area} + P_{area}) * 100$

IS: internal standard; S: substrate; P = product.

3. Supplementary Results

Expressions of OMT3, OMT1, 2-ODD and CYP71CU1

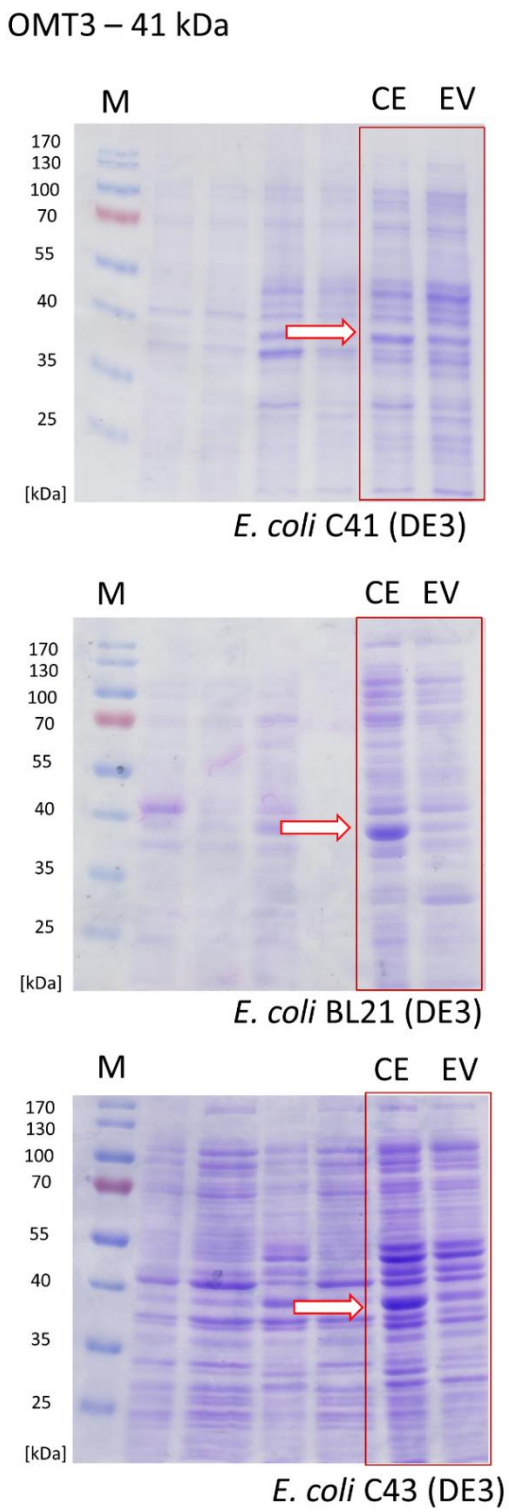


Figure S1. 12.5% SDS-gel of OMT3 (41 kDa; marked by red arrow) expression in various *E. coli* strains. M: Molecular weight size marker, EV: Vector control without *omt3* gene, CE: Soluble protein fraction after cell disruption.

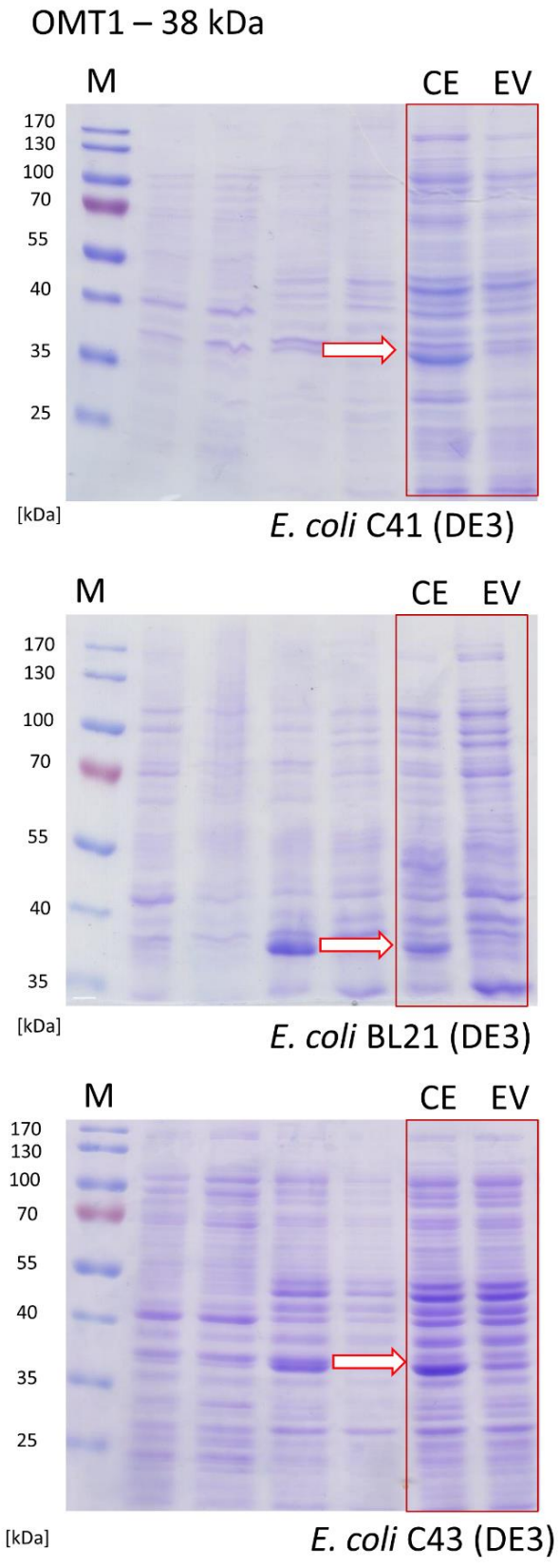


Figure S2. 12.5% SDS-gel of OMT1 (38 kDa; marked by red arrow) expression in various *E. coli* strains. M: Molecular weight size marker, EV: Vector control without *omt1* gene, CE: Soluble protein fraction after cell disruption.

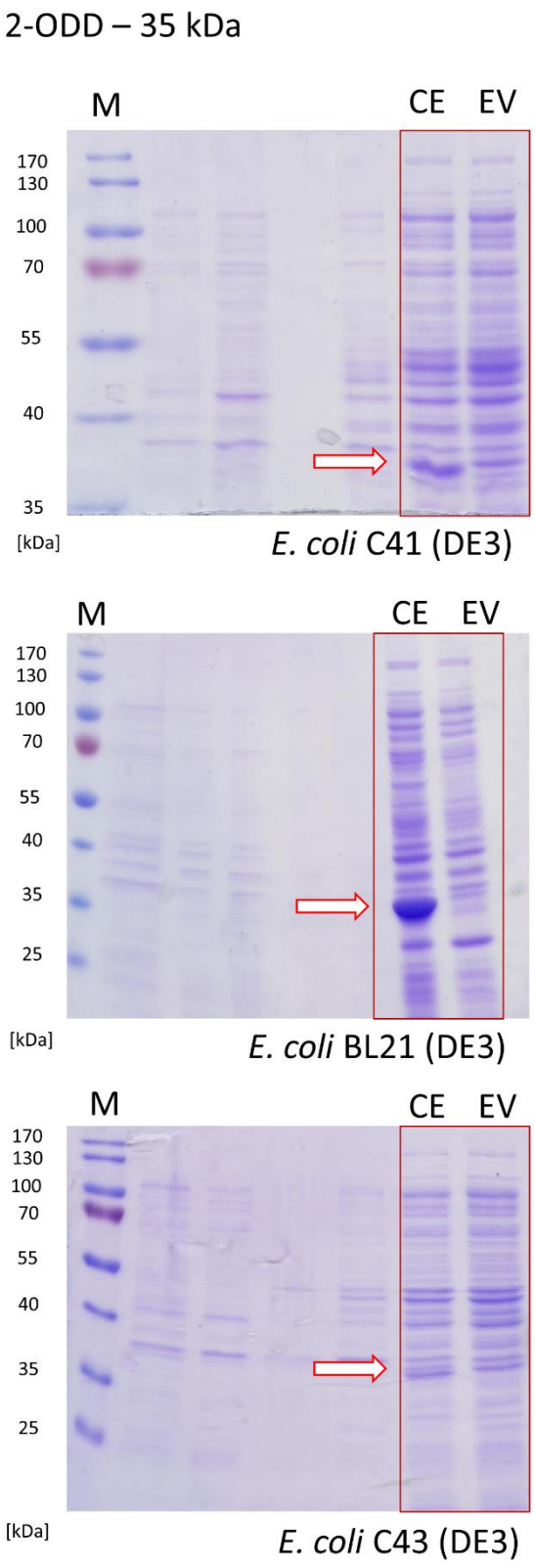


Figure S3. 12.5% SDS-gel of 2-ODD (35 kDa; marked by red arrow) expression in various *E. coli* strains. M: Molecular weight size marker, EV: Vector control without *2-odd* gene, CE: Soluble protein fraction after cell disruption.

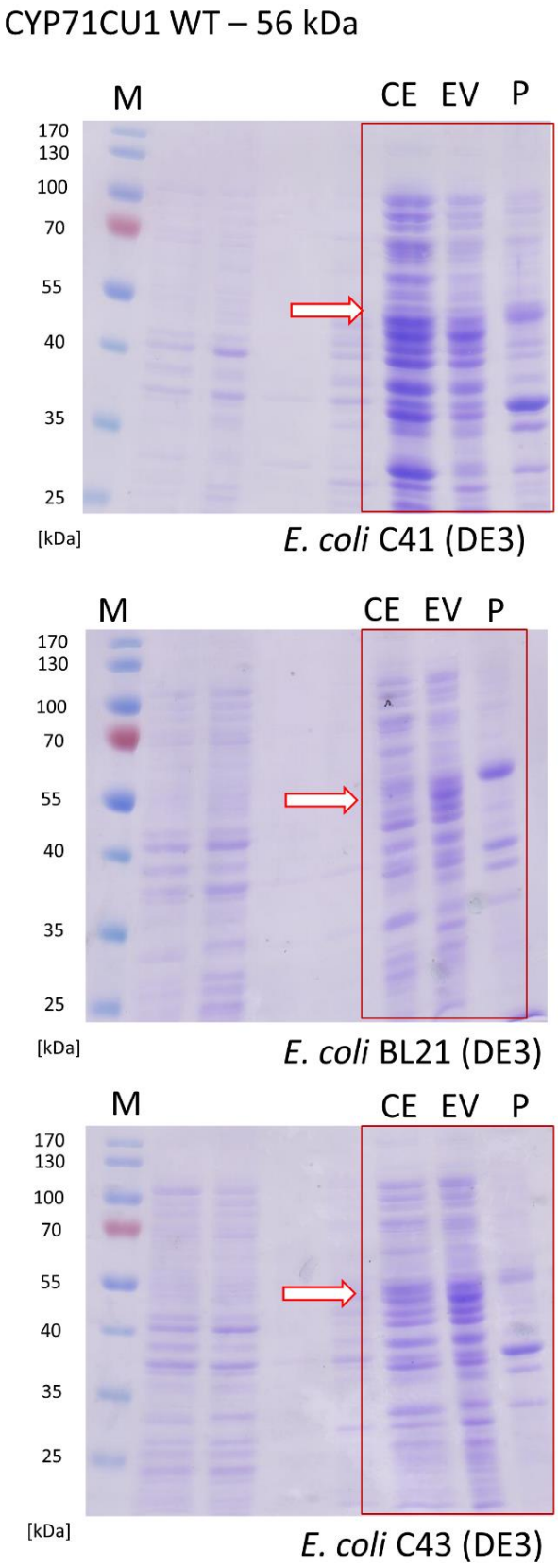


Figure S4. 12.5% SDS-gel of CYP71CU1 (56 kDa; marked by red arrow) expression in various *E. coli* strains. M: Molecular weight size marker, EV: Vector control without *cyp71cu1* gene, CE: Soluble protein fraction after cell disruption, P: Insoluble protein fraction after cell disruption.

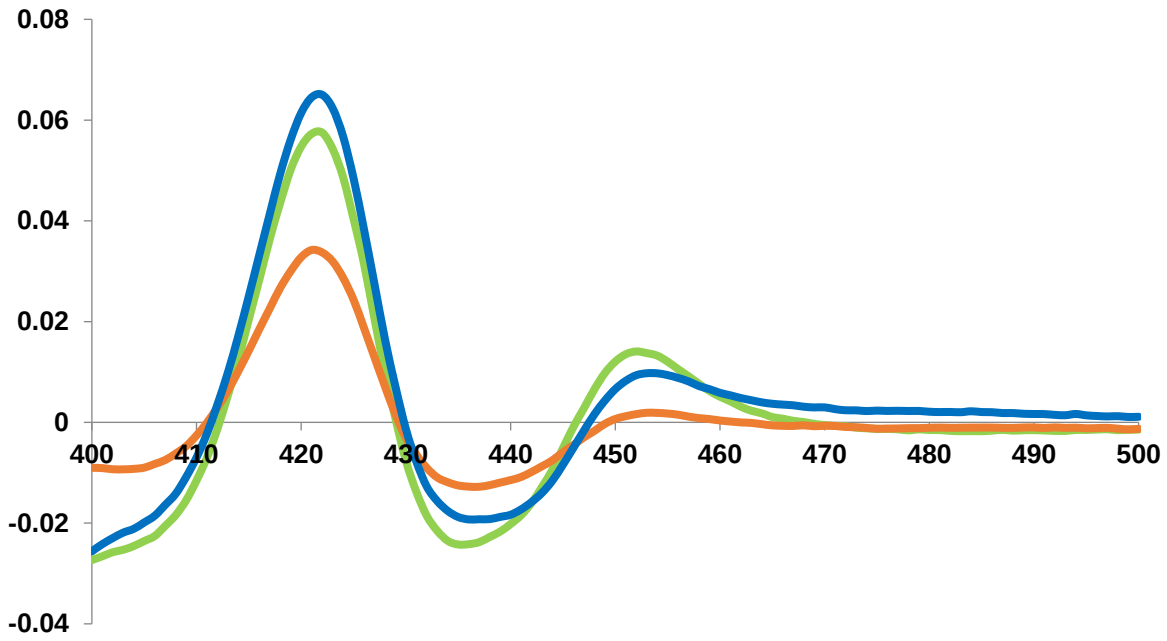


Figure S5. CO-difference spectra of soluble CYP71CU1OC_WT in *E. coli* BL21 (DE3) (orange line), C41 (DE3) (blue line), and C43 (DE3) (green line) after 24 h expression.

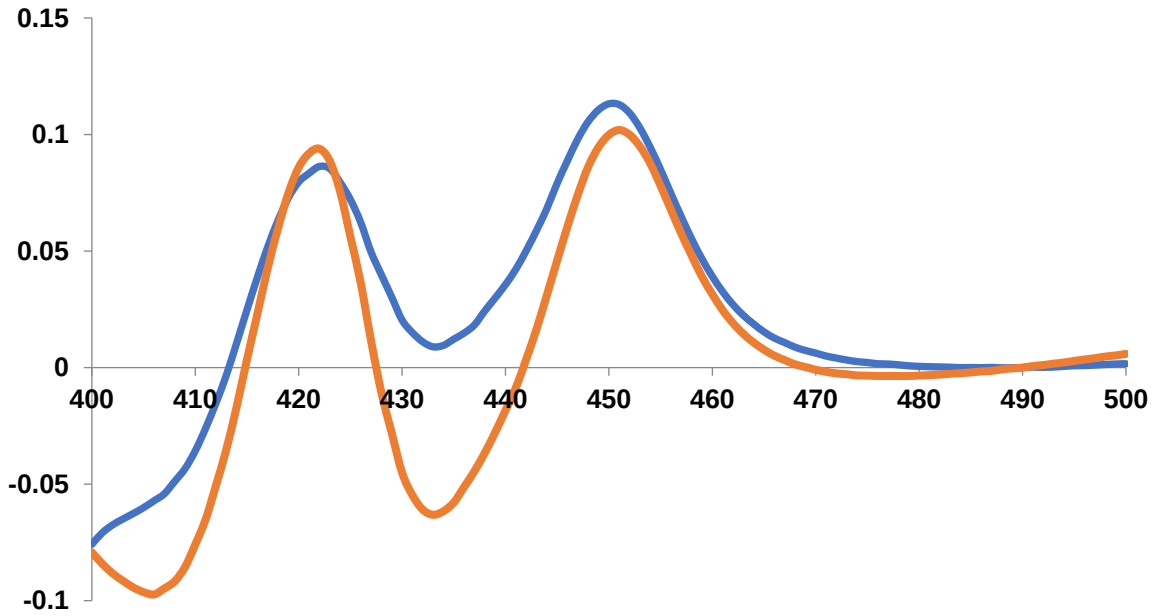


Figure S6. CO-difference spectra of soluble CYP71CU1nat_WT (blue line) and CYP71CU1OC_WT (orange line) after 48 h expression.

Table S8. Expression levels of CYP71CU1 variants in *E. coli* C41(DE3) at 25°C after 48 h.

Variant	Inserted modification	Truncated amino acids	P450 concentration	
			[µg/g_{cww}]	[mg/$l_{culture\ volume}$]
CYP71CU1nat_WT	-	-	263 ± 16	8 ± 5
CYP71CU1Δ20nat	Ala	1 - 20	813 ± 163	26 ± 6
CYP71CU1oc_WT	-	-	159 ± 16	6 ± 1
CYP71CU1Δ20oc	Ala	1 - 20	465 ± 138	15 ± 4

Table S9. Expression levels of N-terminally truncated CYP71CU1 variants in *E. coli* C41(DE3) at various incubation temperatures after 48 h.

Temperature	P450 concentration			
	[µg/g_{cww}]		[mg/$l_{culture\ volume}$]	
	CYP71CU1Δ20oc	**CYP71CU1Δ20nat**	**CYP71CU1Δ20oc**	**CYP71CU1Δ20nat**
20°C	282 ± 26	566 ± 39	11 ± 1	20 ± 3
25°C	465 ± 138	813 ± 163	15 ± 4	26 ± 6
30°C	112 ± 18	253 ± 108	6 ± 3	8 ± 3

Table S10. Expression levels of N-terminally truncated CYP82D61 variants in *E. coli* C41(DE3) at various incubation temperatures after 48 h.

Temperature	P450 concentration			
	[µg/g_{cww}]		[mg/$l_{culture\ volume}$]	
	CYP82D61oc_WT	**CYP82D61Δ23oc**	**CYP82D61oc_WT**	**CYP82D61Δ23oc**
20°C	n.d.	20 ± 17	n.d.	0.7 ± 0.6
25°C	n.d.	936 ± 34	n.d.	33 ± 2
30°C	250 ± 14	1395 ± 220	8 ± 1	42 ± 9

n.d.: Expression not detectable

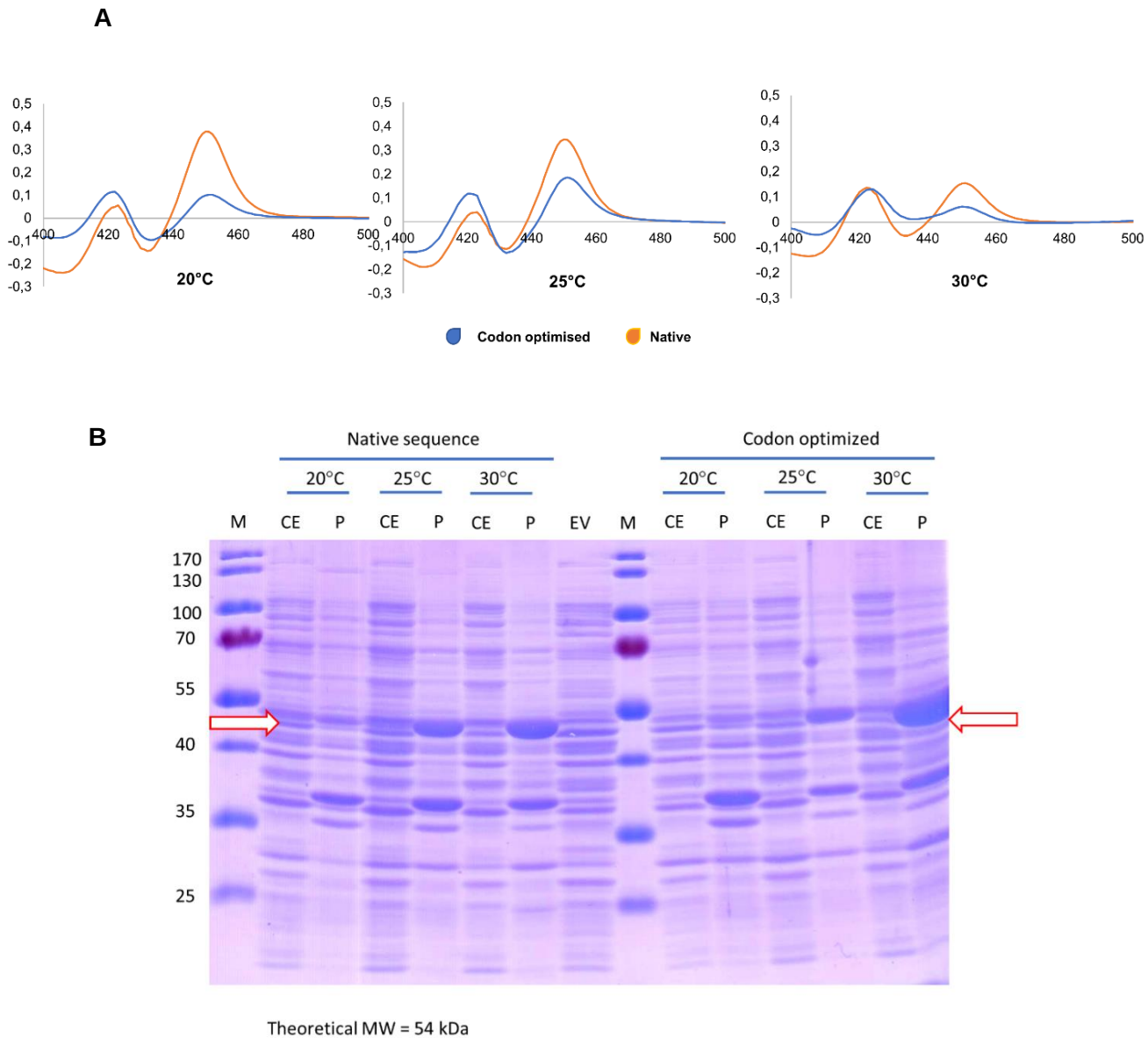


Figure S7. Expression analysis of CYP71CU1 variants incubated at different temperatures. **(A)** CO-difference spectra of CYP71CU1Δ20nat (orange line) and CYP71CU1Δ20oc (blue line). **(B)** 12.5% SDS-gel of CYP71CU1Δ20nat and CYP71CU1Δ20oc expressions (54 kDa; marked with a red arrow). M: Molecular weight size marker, EV: Vector control without *cyp71cu1* gene, CE: Soluble protein fraction after cell disruption, P: Insoluble protein fraction after cell disruption.

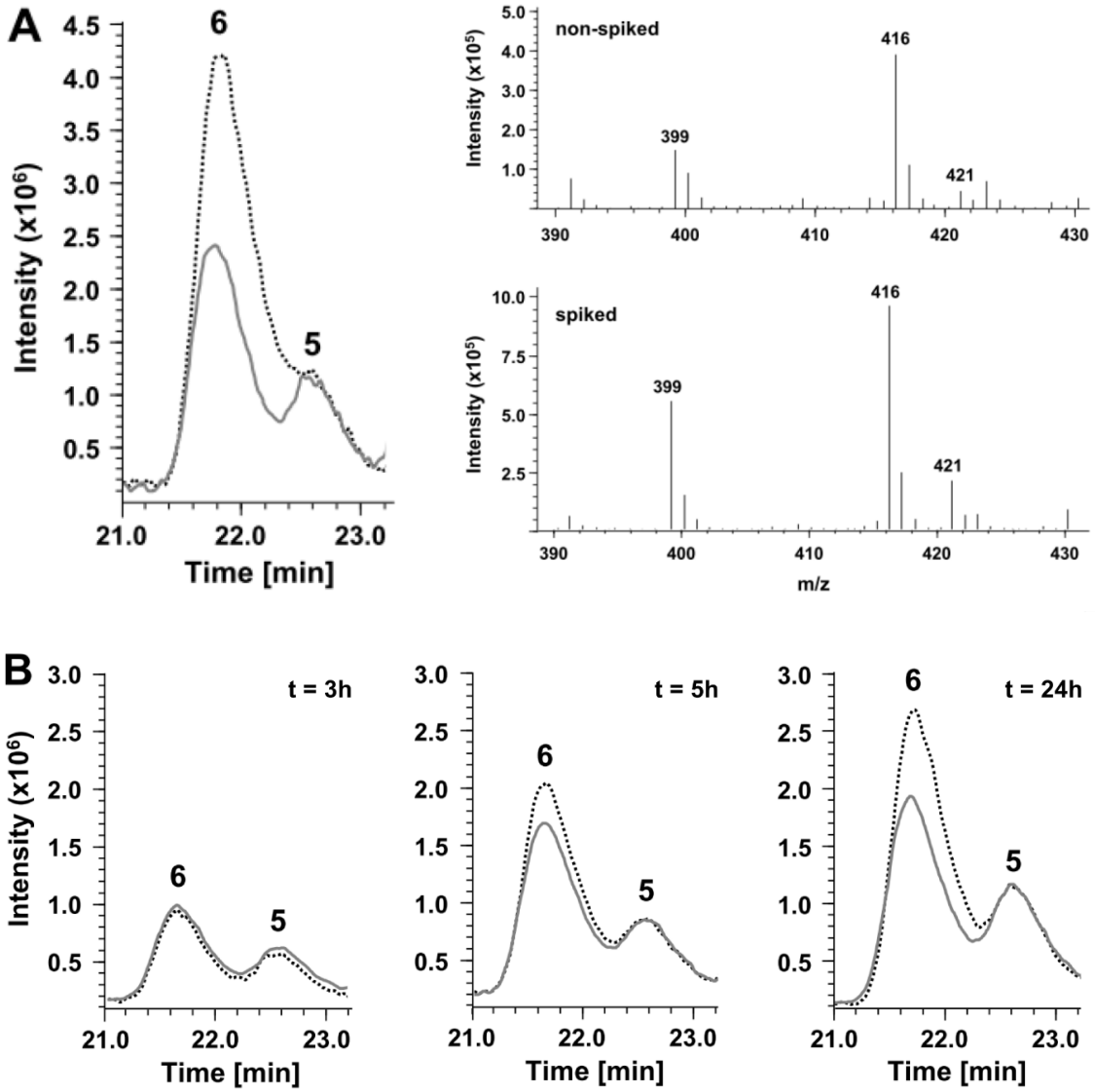


Figure S8. LC/MS analysis (method 4) of one-cell biotransformations of (-)-matairesinol **1** to (-)-deoxypodophyllotoxin **6**. (**A**) LC-chromatogram and m/z-fragmentation at RT = 21.8 min of a reaction sample spiked with 50 µM (-)-deoxypodophyllotoxin **6** (dotted black line) in comparison to the respective non-spiked sample (solid grey line); 399 $[M+H]^+$; 416 $[M+H_2O]$; 421 $[M+Na]^+$. (**B**) (-)-Deoxypodophyllotoxin **6** and (-)-yatein **5** peaks of reaction samples at t = 3 h, 5 h and 24 h with addition of 2.5 mM 2-oxoglutarate at t = 4 h (dotted black lines) in comparison to the respective samples without addition of 2-oxoglutarate (solid grey lines).

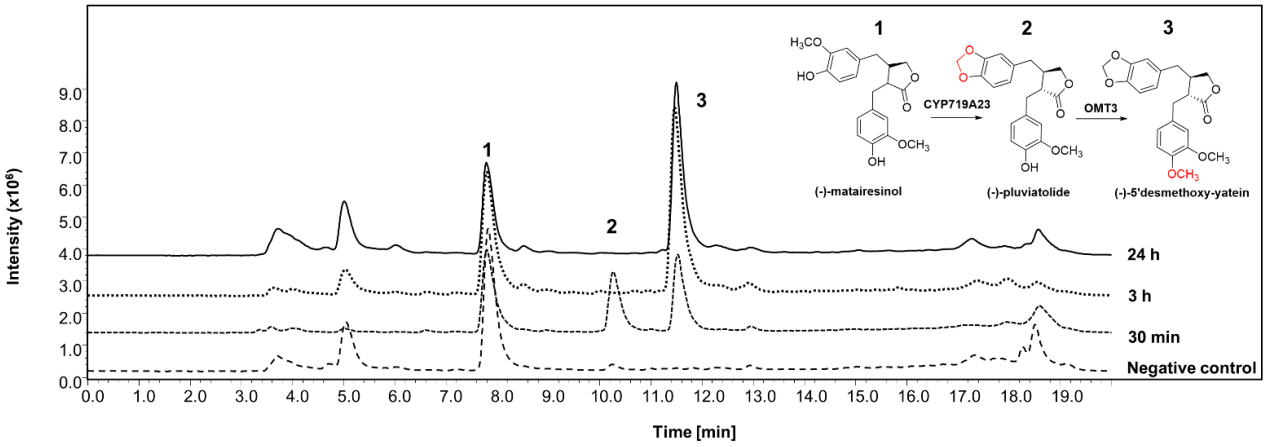
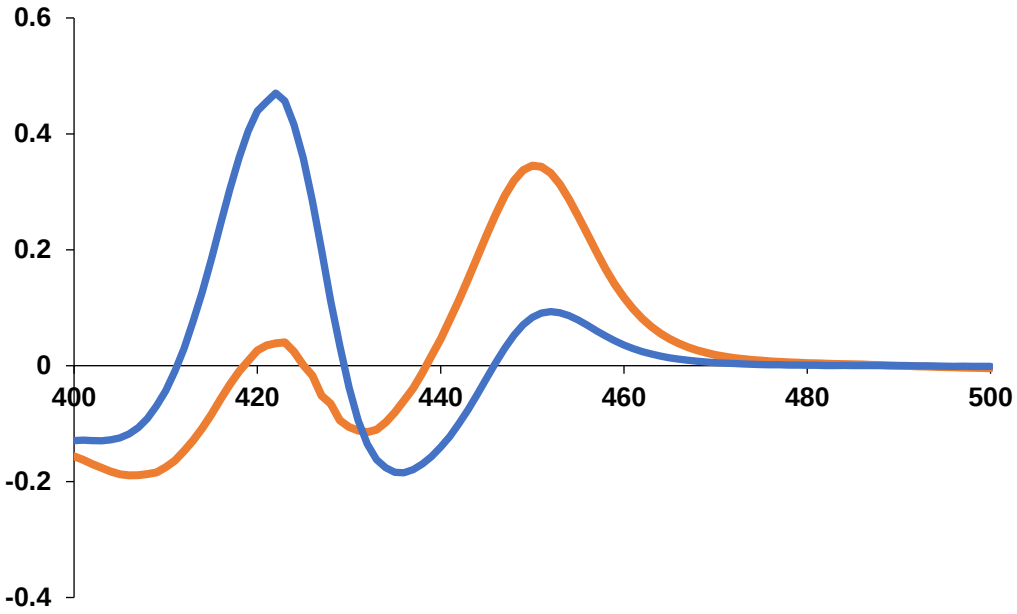


Figure S9. LC/MS analysis (method 1) of OMT3 activity in the cascade reaction with CYP719A23.

A



B

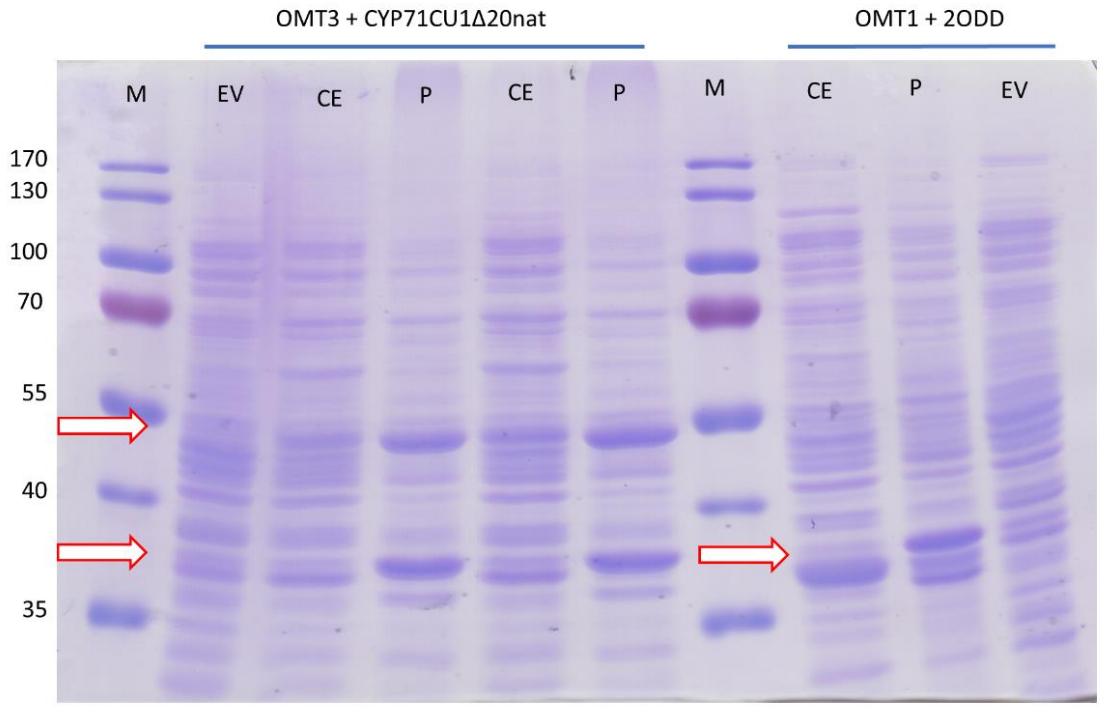


Figure S10. (A) CO-difference spectra of single expressed CYP71CU1Δ20nat (orange line) and CYP71CU1Δ20nat co-expressed with OMT3 (blue line). **(B)** 12.5% SDS-gel of CYP71CU1Δ20nat (54 kDa) co-expression with OMT3 (41 kDa), and OMT1 (38 kDa) with 2-ODD (35 kDa). M: Molecular weight size marker, EV: Vector control without respective genes, CE: Soluble protein fraction after cell disruption, P: Insoluble protein fraction after cell disruption.

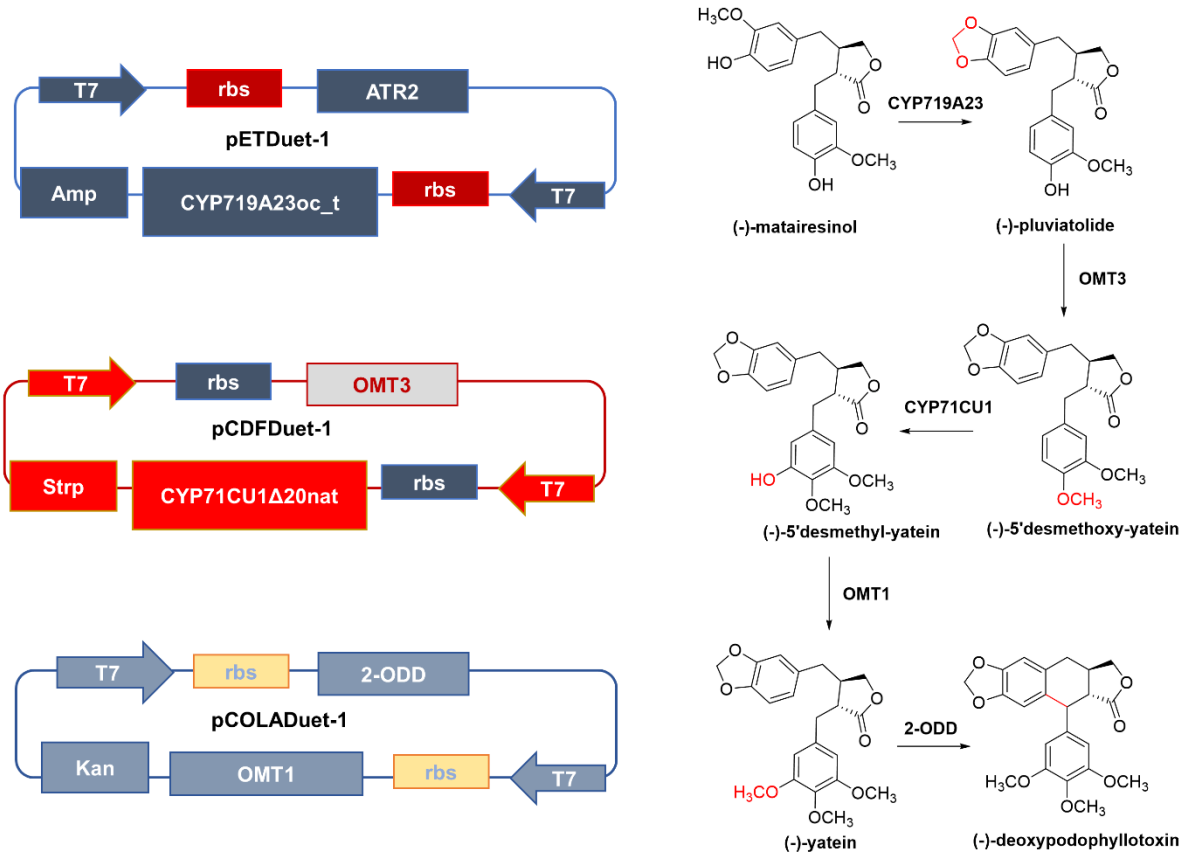


Figure S11. Schematic overview on the plasmid-based modular strategy developed to synthesize (-)-deoxypodophyllotoxin **6** from (-)-matairesinol **1** in *E. coli* via a one-cell 5-steps 6-enzyme reaction cascade.

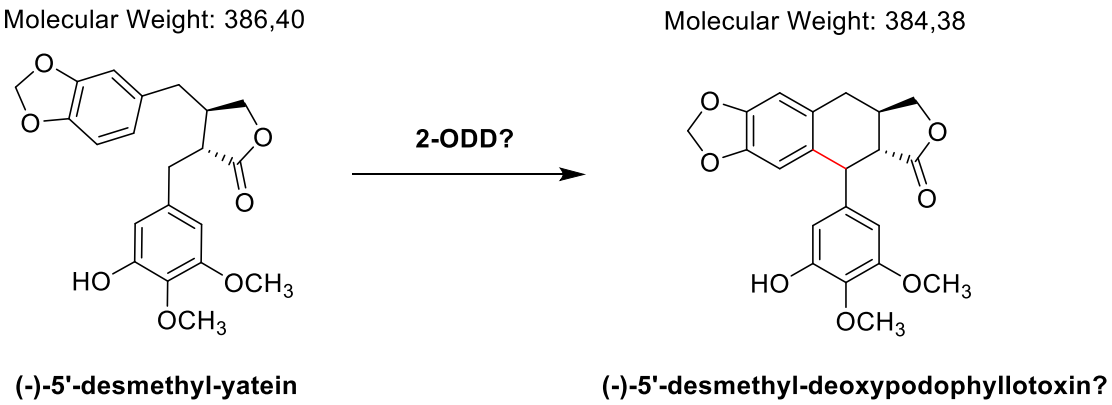


Figure S12. Hypothesized side reaction of 2-ODD occurring in the two-cell approach.

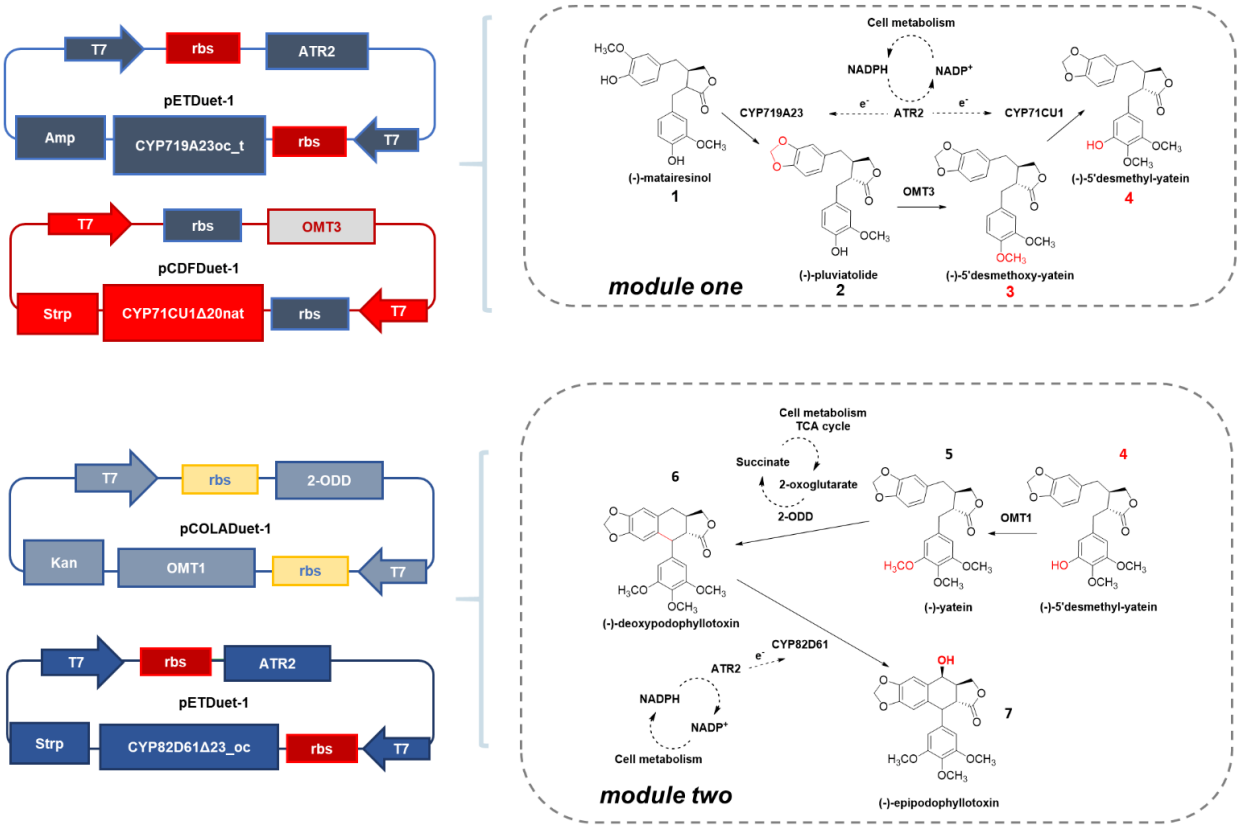


Figure S13 Schematic overview on the plasmid-based modular strategy developed to synthesize (-)-epipodophyllotoxin **7** from (-)-matairesinol **1** in *E. coli* via a two-cell 6-steps 7-enzyme reaction cascade.

4. Supplementary References

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