SUPPLEMENTARY INFORMATION APPENDIX

Consolidated production of coniferol and other high-value aromatic alcohols directly from lignocellulosic biomass

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Materials and methods

Analytical grade reagents and solvents were obtained from Sigma-Aldrich. *Escherichia coli* DH5α and BL21 and BL21 (DE3) cells were purchased from New England Biolabs (Ipswich, MA, USA). Expression vectors pET-28a, pCDF1b and pBbE8kRFP [1] were purchased from Novagen (Darmstadt, Germany) and were used for gene expression. Restriction enzymes, T4 ligase, *taq* polymerase, dNTPs and broad protein marker (2-212 kDa) NEBuilder were purchased from New England Biolabs (Ipswich, MA, USA). The RARE (ADH/AKR knock-out) strain, was purchased from addgene [2]. Microbiological media ingredients were obtained from Formedium™ (LB and TB) and prepared according to the recommended protocols provided. The Insoluble wheat arabinoxylan (Megazymes) consisted in a high purity arabinoxylan (~ 80%) carefully extracted and purified to maintain the ferulic acid crosslinks in the native arabinoxylan. Composition: (Ara: Xyl = 36: 51, Glucose 6.5%, mannose 4.4% and galactose 1.6%). The wheat straw (WS) was provided by Biopower technologies ltd (Poland). The material was collected in the 2016 crop with D_{50}<40 µm. All the plasmids and genes constructs used in this work are displayed in Supplementary Table S2.

DNA cloning - whole cell biocatalysis To convert ferulic acid (**1**) to coniferyl alcohol (**3**) we constructed a variety of the biotransformation plasmids. First, the construct pZZ-Eva2, built from pBbE8kRFP [1] and reported by Klumbys et al (2018) [3] was used for the construction of the final plasmid pROB1 (Supplementary Fig.7). These constructs contained the most genes/parts (CgAKR-1/KRED, SRCAR/NiCAR, Sfp and P-Rham/ P-Ara) previously reported) [3]. From pZZ-Eva2 we built new plasmids removing the phenylalanine lyase present in this plasmid, followed by removing or substituting KRED-1 present for CgAKR-1, and substituting SrCAR for NiCAR (pROB1) (Supplementary Table 2). For the construction of the plasmids, each part was separately amplified from the corresponding expression vector by polymerase chain reaction (PCR) using the Phusion-polymerase. Finally, inverse PCR was employed to linearize the plasmid backbone. The PCR product was treated with DpnI (1 hour, 37 °C). All parts and the backbone were fused together to form a single vector in a molar ratio 2:1 using the *NEBuilder HiFi DNA Assembly® Master Mix* (NEB). The plasmid insert region, which represents the bioengineered pathway, was fully sequenced by Sanger sequencing. The KRED/CgAKR-1-SrCAR/NiCAR operon is under the control of an L-arabinose inducible promoter, and Sfp is under the control of an L-rhamnose inducible promoter. The plasmids encode kanamycin resistance gene and a colE1 origin of replication with 20-30 copies per cell.

Enzyme expression and purification The *E. coli* strain BL21 (DE3) (For CARs and XynZ) or ArcticExpress (DE3) (For CgAKR-1) competent cells were transformed with the pET-28a plasmid containing the designed genes and plated in selective solid LB medium containing kanamycin (50 mg/L). For expression of CAR enzymes, cells were transformed with the pCDF1b vector (Novagen), containing the Phosphopantetheinyl transferase from *Bacillus subtilis,* and transformants were selected with streptomycin 50 mg/L on plates. After, a single colony was used to inoculate liquid LB containing kanamycin 50 mg/L and streptomycin 50 mg/L for 16 h at 37 °C and 200 rpm. The cultures were then diluted in 600 mL fresh LB medium containing kanamycin and streptomycin when pCDF1b vector was present in the strain, and grown at 30 °C and 200 rpm for 4 h. The expression of the recombinant proteins was induced by the addition of 1 mM isopropyl-d-1-thiogalactopyranoside (IPTG) followed by incubation for 18 hours at 30°C for BL21(DE3) at 12 C° for ArcticExpress (DE3). *E. coli* cells were harvested and re-suspended in 100 ml 50 mM potassium phosphate buffer pH 8.0, 500 mM NaCl and 10 mM imidazole buffer. Cells were disrupted by the French Press at 1500 psi and cell debris removed by centrifugation at ~48,000 x g, 4 °C for 1 h. Cell lysate was filtrated using 0.45 µm Minisart NML syringe filters (surfactant free cellulose acetate membrane) and loaded on Ni-IDA resin (Generon). Stepwise elution performed with 50 mM potassium phosphate buffer pH 8.0, 100 mM NaCl with increasing concentration of imidazole (50-250 mM) buffer at 4°C. The sample after Ni-IDA was concentrated by the combined use of Amicon stirred cell concentrator (76 mm, 100,000 MWCO polyethersulfone membrane Discs from generon) and Vivaspin 20 (100,000 MWCO PES membrane GE Healthcare) at 2,800 x g up to 6 ml at 4 °C. 3 ml was loaded onto the HiLoad 16/60 Superdex 200 with 5 ml loop and isocratically eluted in 50 mM potassium phosphate buffer pH 7.5, 150 mM NaCl, 2mM MgCl at 1 ml/min flow rate at 4 °C. Eluent with high A_{280} absorbance was collected and concentrated using a Vivaspin 20 centrifugation tube (100,000 MWCO PES membranes from GE Healthcare) at 2,800 x g (4°C). The retained enzyme isolate was then frozen as several protein ball stocks by dropping protein solution slowly in liquid nitrogen for storage at -80 °C.

***In-vitro* assays** Assays were performed in order to evaluate the biotransformation of **1** by a set of 5 recombinant CARs, alone or in cascade combination with CgAKR-1. The procedure was prepared as following: 1 mM **1**, 6 mM NADPH, 10 mM ATP, 10 mM $MgCl_2$, 100mM Tris-HCl pH 7.5, 250µg CAR alone or in combination with 100 µg CgAKR-1 in a final volume of 500 µl. Samples were taken after 18 hours of incubation. In sequence, a time course experiment was performed using NiCAR and SroCAR with or without CgAKR-1 over 18 hours. All The reactions were performed at 30 ^{o}C, 250 rpm in 2 mL tubes (positioned vertically) and were monitored by LCMS.

CgAKR-1 enzymatic assay AKR activity was assayed spectrophotometrically at 30 °C by monitoring the decrease in the absorbance of NADPH at 340 nm in microplates (Supplementary Fig. 1). The standard assay mixture (0.2 mL) was composed of 50 mM sodium phosphate buffer (pH 7.0), 5.0 mmol/L substrate (2-nitrobenzaldehyde, furfural, HMF, etc.), and 2.5 µg CgAKR-1. Additionally, 0.2 mmol/L NADPH was added to the plate to initiate the reaction, and the reaction rate was measured against an identical blank with no enzyme added. Activity was measured for 5 min. One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation of 1 µmol NADPH per minute (µmol/mg/min) under the described assay conditions. The activity on 2-nitrobenzaldehyde was used as reference for the comparison using other aldehyde substrates and expressed in relative activity (%).

Whole cell biocatalysis BL21 and RARE *E. coli* strains were transformed with the constructed plasmids (Supplementary Table 3) in order to evaluate the whole cell biotransformation of **1** to coniferyl aldehyde (**2**) and **3.** The *RARE* strain was used because it lacks several ADHs/AKRs capable to reduce **2** which allows us to compare the capability of aldehyde reduction from the heterologous versus endogenous *E. coli* system [2]. In order to prepare the inoculum for the whole cell production experiment, one single colony of *E. coli* transformed with the appropriate vectors, was picked, transferred into 15 ml falcon tubes and grown in 2000 µL of TB media supplemented with 50 mg L^{-1} kanamycin overnight at 37°C while shaking at 200 rpm. 10 ml of TB media was then inoculated with 1% of the starting inoculum and incubated at 30°C for 4h at 200 rpm. Initially, all cultures were induced with 5 mM L-arabinose and 5 mM L-rhamnose and grown for more 4 hours. After gene expression, 5 mM of **1** was added in the media and samples were harvested over different times after induction to test **3** production monitored by LCMS. Optimization of Experiments was performed on the biotransformation process using a full 2^3 factorial design leading to 8 sets of experiments with four replicates at central point was used to verify the most significant factors affecting **3** production. The designed range and the level of the variables investigated in this work were listed in Supplementary Table 1. The productivity of **3** was considered as the response (Y). To stop the reaction, the cultures were centrifuged for 10 min at 10,000 x g.

Scale up procedure and compound characterization The optimized conditions (Supplementary Table 1) were used to scale-up the biotransformation to 100 mL with 5 mM of ferulic acid substrate. Following the biotransformation, the product was isolated by standard and slightly modified previously reported procedures [3]. Briefly, the cell culture media was centrifuged at x 10,000 x g for 10 min, to remove the *E. coli* cells, the remaining culture media supernatant was added to 30 mL of brine, and the aqueous solution was extracted with (4 x 20 mL) ethyl acetate (EtOAc). The combined organic phases were dried using anhydrous $MgSO_4$, filtered, and the solvent removed *in vacuo* using

a rotary evaporator. This afforded an oily crude residue 102 mg containing 74% coniferyl alcohol and 26% coniferyl aldehyde (assessed by NMR) before purification. The crude mixture was then purified via silica-gel flash column chromatography Cyclohexane: EtOAc (8:2 then 7:3) to afford the purified coniferyl aldehyde (18 mg, 78% isolation yield) and coniferyl alcohol product (59 mg, 88% isolation yield). The isolated products were characterised by ^{1}H and ^{13}C NMR and high-resolution mass spectrometry (Supplementary Fig. S4 and Supplementary Fig. S5).

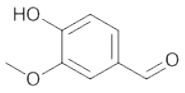
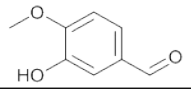
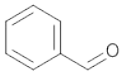
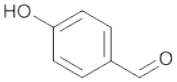
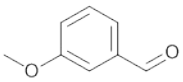
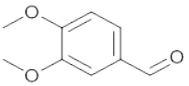
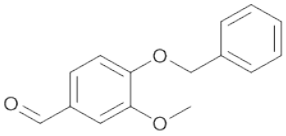
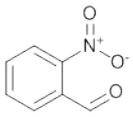
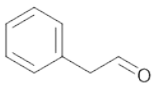
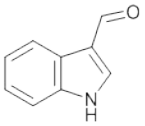
Lignocellulose degradation and biotransformation This step was performed as already described in the section above with the following differences: Wheat arabinoxylan (WAX) or wheat straw (WS) 2 0g/L (2% w/v) and 1 mg/g substrate of purified feruloyl esterase - XynZ were added in the cell culture after the expression phase instead of **1**. The aromatic components were purified, concentrated, and derivatized: 400 µL of the biotransformation or chemical standards were added with 25 µL 1 M HCl and 400 µL EtOAc and vortexed vigorously for 1 minute. The solution was centrifuged for 5 min at 10,000 x g and the organic layer was transferred to a 1.5 mL tube. This step was repeated to ensure total phenolic extraction. The solvent was removed by speed-vac and the resulting dry material was dissolved in milli-Q water for posterior quantification by LCMS. No residual phenolics were detected in the aqueous phase.

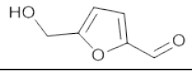
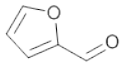
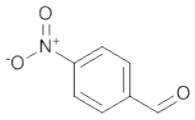
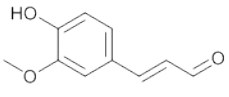
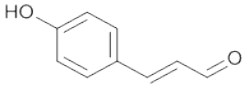
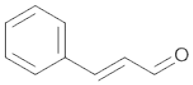
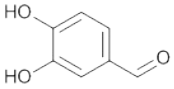
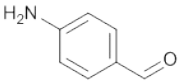
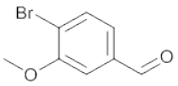
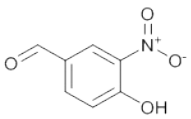
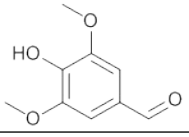
LC/MS analysis In order to analyze the phenolic compounds after the biotransformation, reverse phase HPLC was performed on an Agilent 1200 Series LC system equipped with a G1379A degasser, a G1312A binary pump, a G1329 autosampler unit, a G1316A temperature controlled column compartment and a G1315B diode array detector. Final reaction solutions were transferred to a Thomson Standard Filter Vial for samples microfiltration and processed on LC using standards (Sigma). Conversion for the catalyzed reaction was calculated from reverse phase liquid chromatography performed using a HiChrom ACE 5 C18-AR column (15 cm x 4.6 mm). Separation conditions: 1 mL min^{-1} flowrate, mobile phases: H_2O + 0.1% v/v TFA, MeOH + 0.1% v/v TFA. Gradient: 95:5 for 4 min, to 0:100 over 25 min. Temperature: 30 °C. Detection wavelength: 210 nm. Injection volume: 10 µL. Compounds were ionized using API-electrospray technique and detected in positive mode on the LCMS System. Drying gas temperature 250 °C at 12 L min^{-1}, and nebulizer pressure at 25 psig. All products, unless otherwise specified, were identified by their $[M+H]^{+}$ signal and confirmed via chemical standards.

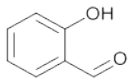
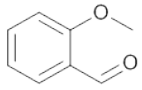
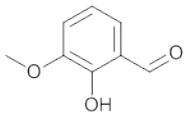
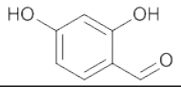
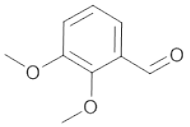
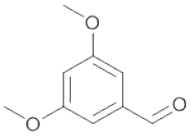
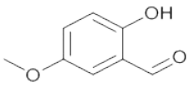
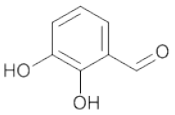
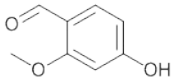
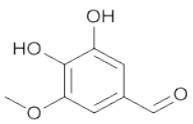
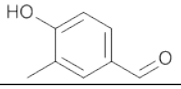
GC-MS Analysis All the reaction products were primarily analyzed by GC-MS using an Agilent Technologies 7890B GC equipped with an Agilent Technologies 5977A MSD. To the biotransformation reaction 50 µL of 5M HCL was added and the products were extracted with 400 µL MTBE. The organic layer was dried over $MgSO_4$ and transferred to a HPLC vial (with insert) through a filtered tip. The products were derivatised by adding 20 µL BSTFA + 1% TMCS and incubated at 50 °C for 30-60 mins. The products were separated on a DB-WAX column (30 m x 0.32 mm i.d., 0.25 µM film thickness, Agilent Technologies). The injector temperature was set at 240 °C with a split ratio of 20:1 (1 µL injection). The carrier gas was helium with a flow rate of 2 mL/min and a pressure of 4.6 psi. The following oven program was used: 100°C (0 min hold), ramp to 20°C at 4 °C/min (0 min hold), and ramp to 240 °C at 20 °C/min (1 min hold). The ion source temperature of the mass spectrometer (MS) was set to 230 °C and spectra were recorded from m/z 50 to m/z 250. Compound identification was carried out using authentic standards and comparison to reference spectra in the NIST library of MS spectra and fragmentation patterns.

High resolution MS (HRMS) Analysis was performed using waters LCT time-of-flight mass spectrometer connected to a Waters Alliance LC (Waters, Milford. MA. USA).

Supplementary Data Figures and Tables

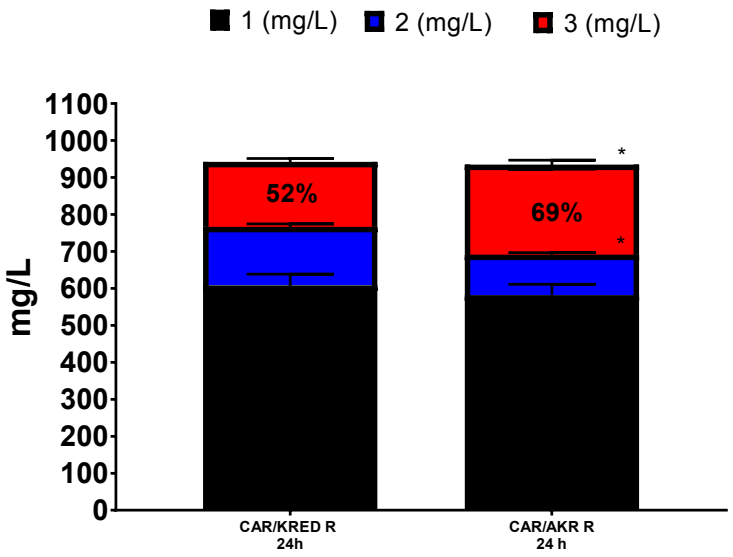
Num.	Substrate	Structure	Rel.%
1	Vanillin (4-Hidroxi-3-metoxibenzaldehyde)		0
2	3-Hydroxy-4-methoxybenzaldehyde (isovanilin)		119
3	Benzaldehyde		250
4	4-Hydroxybenzaldehyde		11
5	3-Methoxybenzaldehyde		76
6	3,4-Dimethoxybenzaldehyde		0
7	4-Benzyloxy-3-methoxybenzaldehyde		0
8	2-Nitrobenzaldehyde		100
9	Phenylacetaldehyde		0
10	Indole-3-carboxaldehyde		0

11	**5-Hydroxymethylfurfural**		**174**
12	**Furfural**		**190**
13	**4-nitrobenzaldehyde**		**6**
<u>14</u>	**<u>Coniferyl aldehyde (2)</u>**		**107**
15	**Coumaryl aldehyde**		**80**
16	**Cinnamyl aldehyde**		**90**
17	**3,4-Dihydroxybenzaldehyde**		**139**
18	**4-aminobenzaldehyde**		**0**
19	**4-Bromo-3-methoxybenzaldehyde**		**0**
20	**4-Hydroxy-3-nitrobenzaldehyde**		**0**
21	**Syringaldehyde**		**74**

22	**2-Hydroxybenzaldehyde**		**11**
23	**2-Methoxybenzaldehyde**		**0**
24	**2-Hydroxy-3-methoxybenzaldehyde**		**615**
25	**2,4-Dihydroxybenzaldehyde**		**0**
26	**2,3-Dimethoxybenzaldehyde**		**550**
27	**3,5-Dimethoxybenzaldehyde**		**878**
28	**2-Hydroxy-5-methoxybenzaldehyde**		**0**
29	**2,3-Dihydroxybenzaldehyde**		**378**
30	**4-Hydroxy-2-methoxybenzaldehyde**		**0**
31	**3,4-Dihydroxy-5-methoxybenzaldehyde**		**0**
32	**4-Hydroxy-3-methylbenzaldehyde**		**0**

33	**Acetaldehyde**		**59**
34	**Glutaraldehyde**		**98**

Supplementary Figure S1. CgAKR-1 relative substrate specificity (%). Specific activity was determined as µmol of NADPH oxidized per min per mg protein. Relative activities of CgAKR-1 using different substrates are presented as the percentage of CgAKR-1 activity on 2-nitrobenzaldehyde. The use of CgAKR-1 in biocatalysis was unknown; therefore, this is the first detailed substrate characterization of an insect AKR with a unique activity range.

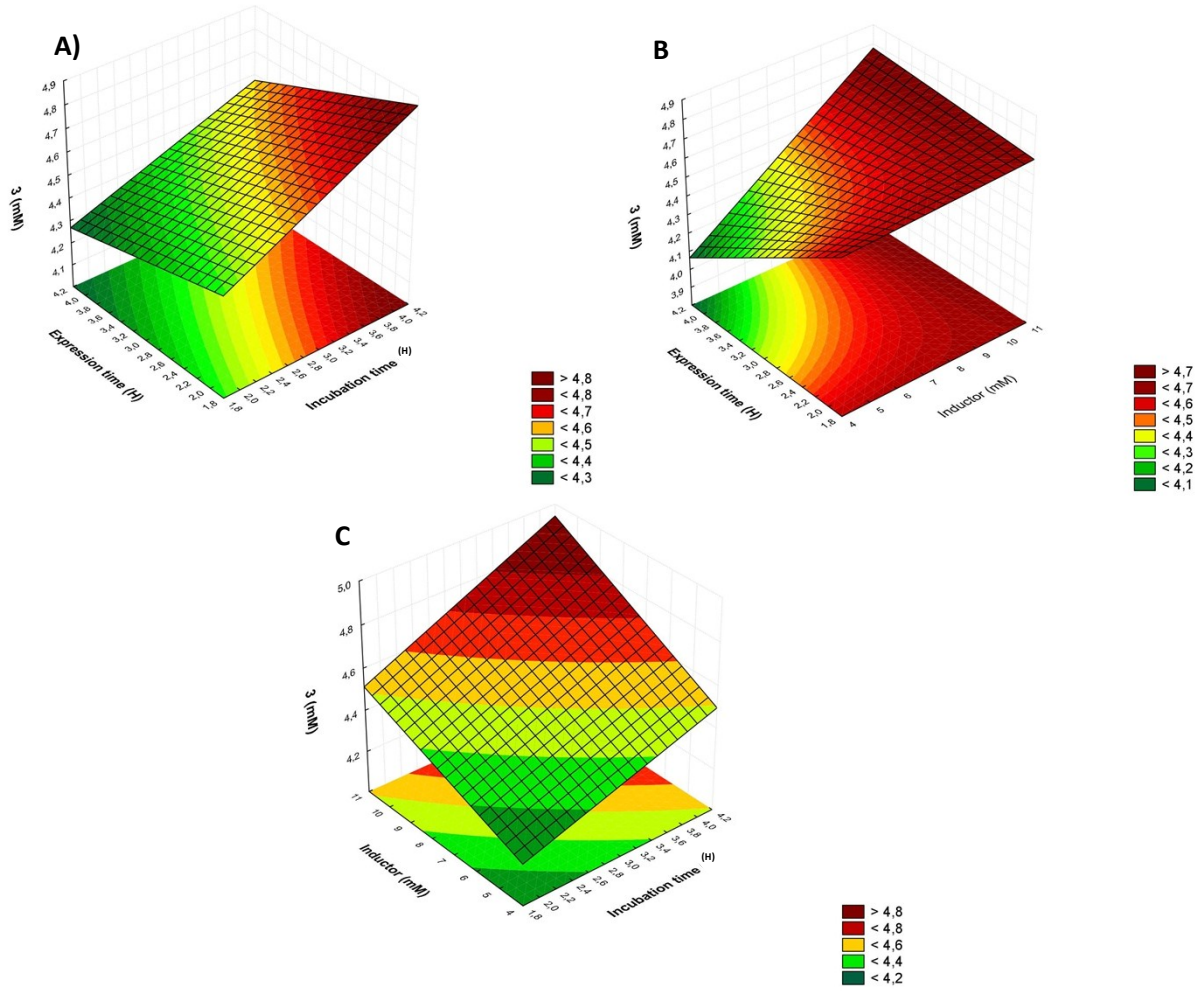


Supplementary Figure S2. Quantification of whole cell biocatalytic activity to produce **2** and **3** from **1** at 24h after in *E coli* RARE strains used transformed with DNA plasmids encoding SRCAR-CgAKR-1 or SRCAR-KRED-1. The results are given in mg/L of medium supplemented with 5mM substrate **1.** In order to evaluate CgAKR-1 capability to integrate the synthetic route for coniferyl alcohol production, we decided to compare the *in-vivo* biotransformation efficiency towards *Paraburkholderia phytofirmans* alcohol dehydrogenase (KRED1) described by Klumbys et al (2018) [3]. Our results, indicates that both enzymes have good activity under the conditions to be used in this system. In general both KRED-1 and CgAKR-1 were able to improve SrCAR performance for the conversion of **1** to **2** and **3** where CgAKR-1 was slightly better for the conversion of **2** to **3**. All the assays were performed in duplicate, * the p-value was <0.05 according to the student test.

Supplementary Table S1. Design of the complete experimental design (DOE) 2^3 for the biotransformation of **1** to **3**.

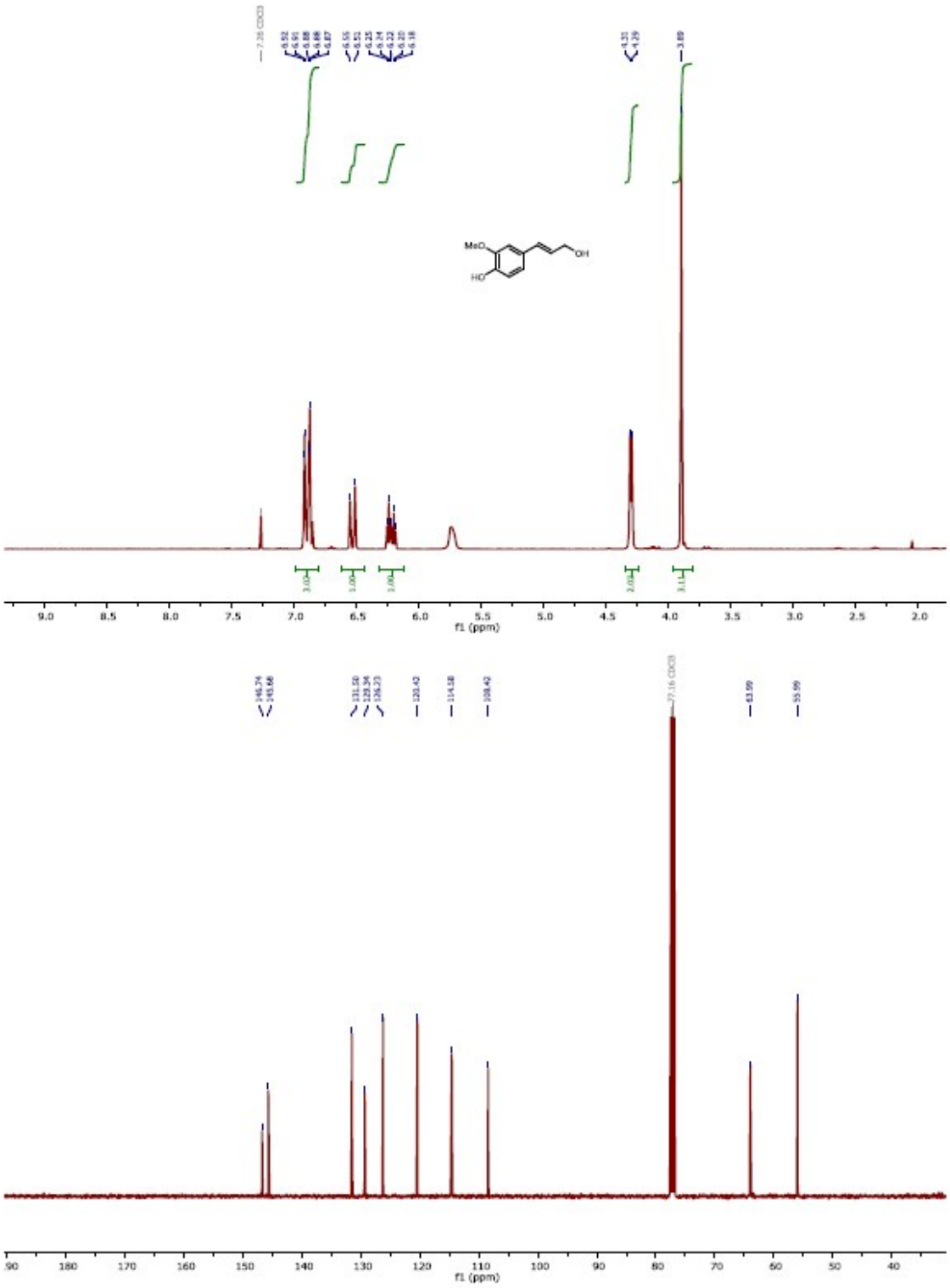
Experiment	Incubation time (hours)	Ara + Rham addition (mM)	Expression time (hours)	1 to 3 conversion (%)	Residual 2 (%)
1	2	5	2	90.9	2.6
2	4	5	2	96.4	3.3
3	2	10	2	91.6	3.8
4	4	10	2	96.6	0
5	2	5	4	87.6	6.5
6	4	5	4	91.0	5.4
7	2	10	4	92.8	3.0
8	4	10	4	95.8	1.1
9 (C)	3	7.5	3	95.4	1.5
10 (C)	3	7.5	3	93.2	0.9
11 (C)	3	7.5	3	94.7	1.1
12 (C)	3	7.5	3	92.2	1.2

The effect as reported % relative percentage of **1** conversion to **3** after 4 hours of **1** addition. All the statistical parameters confirmed the fitness of the model with high significance (R2 of 91% and calculated F value: 23.1> tabled F: 2.84).

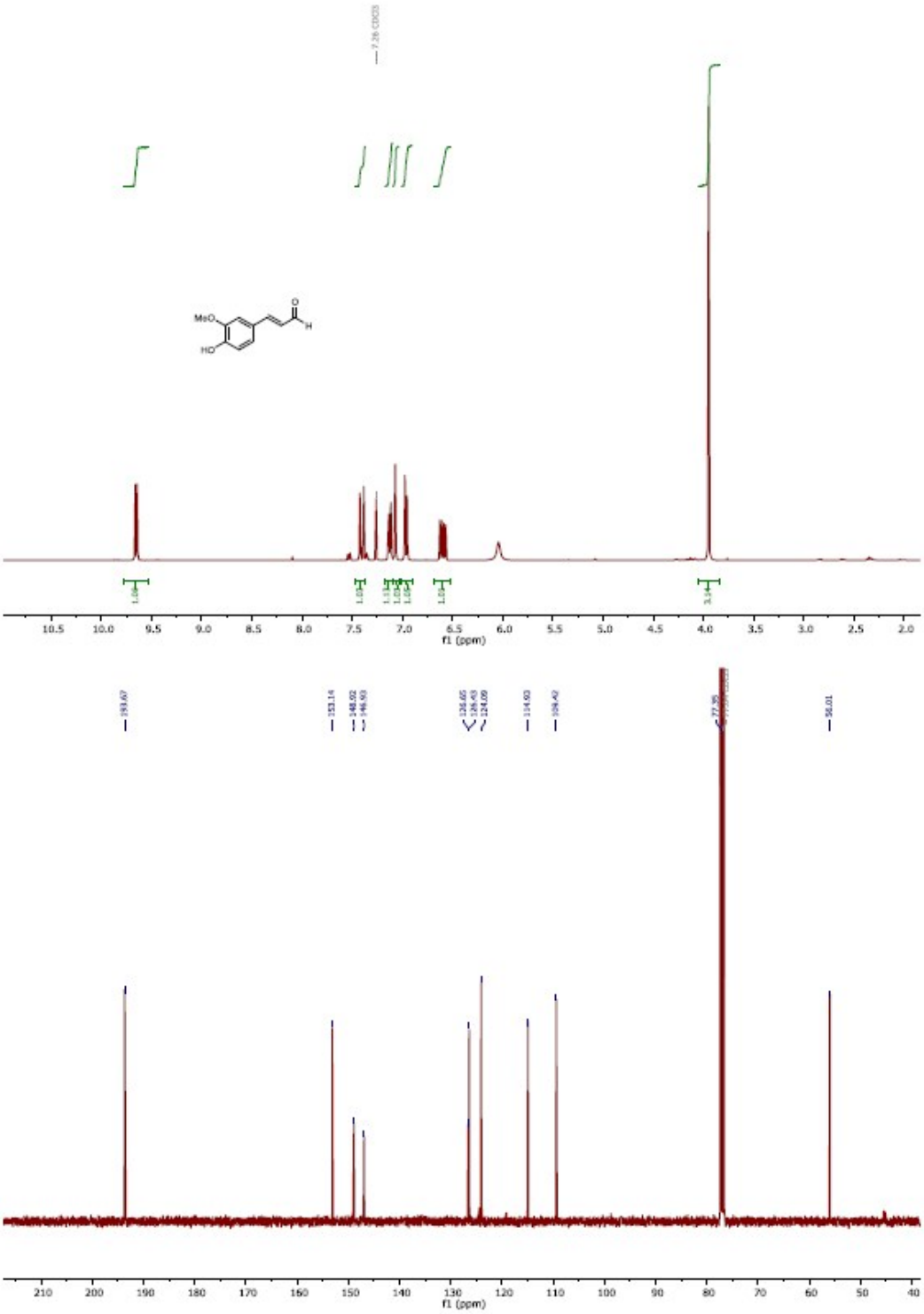


Supplementary Figure S3. Response surface curve for **3** production varying *E. coli* BL21 (NiCAR/CgAKR-1) incubation time before induction (h), Inducer concentration (mM) and expression time before 1 addition (h). The reaction occurred for 4 hours and samples were measured according to S1. **A -** Incubation versus expression time. **B** Inducer concentration versus expression time. **C** Incubation versus inductor concentration. The not interacting variants were set on central values.

Purified Coniferol **3**

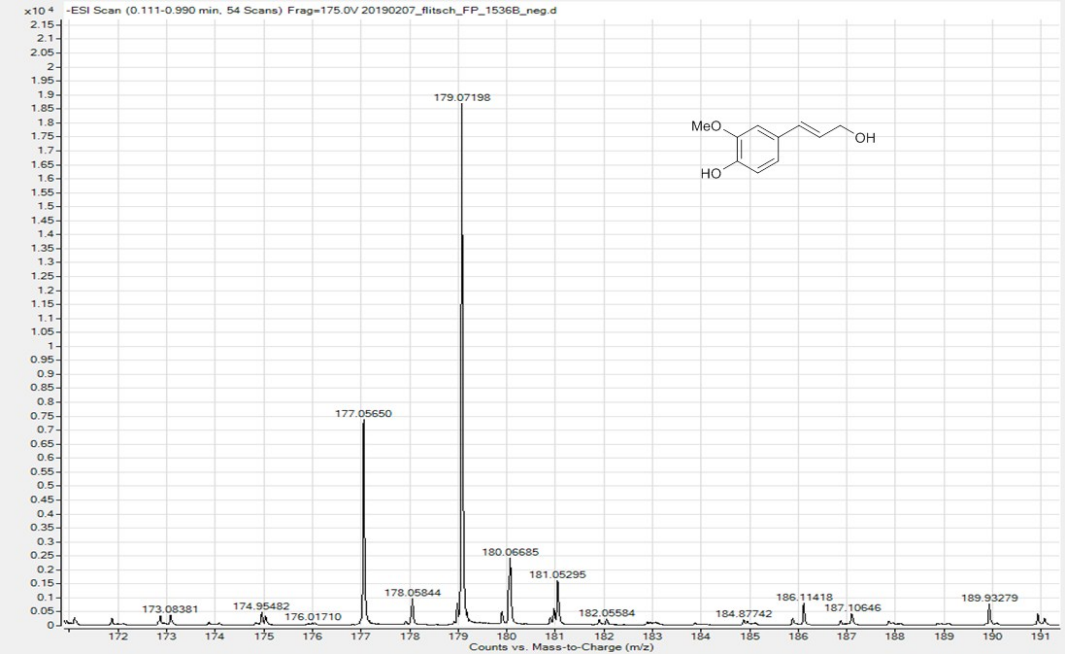


Purified coniferyl aldehyde **2**

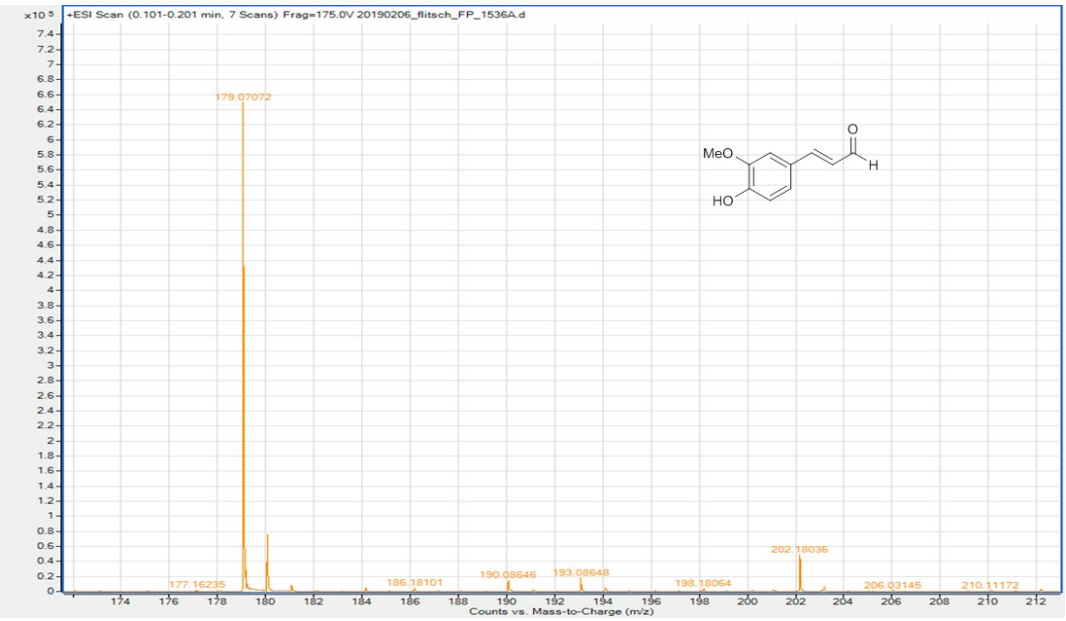


Supplementary Figure S4. Characterization of purified coniferol **3** and coniferyl aldehyde **2** isolated following whole-cell biocatalytic production using the whole cell NiCAR-CgAKR-1 route. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400.1 MHz) without additional internal standard. Chemical shifts are reported as δ in parts per million (ppm), calibrated against residual solvent signal.

Purified Coniferol **3**

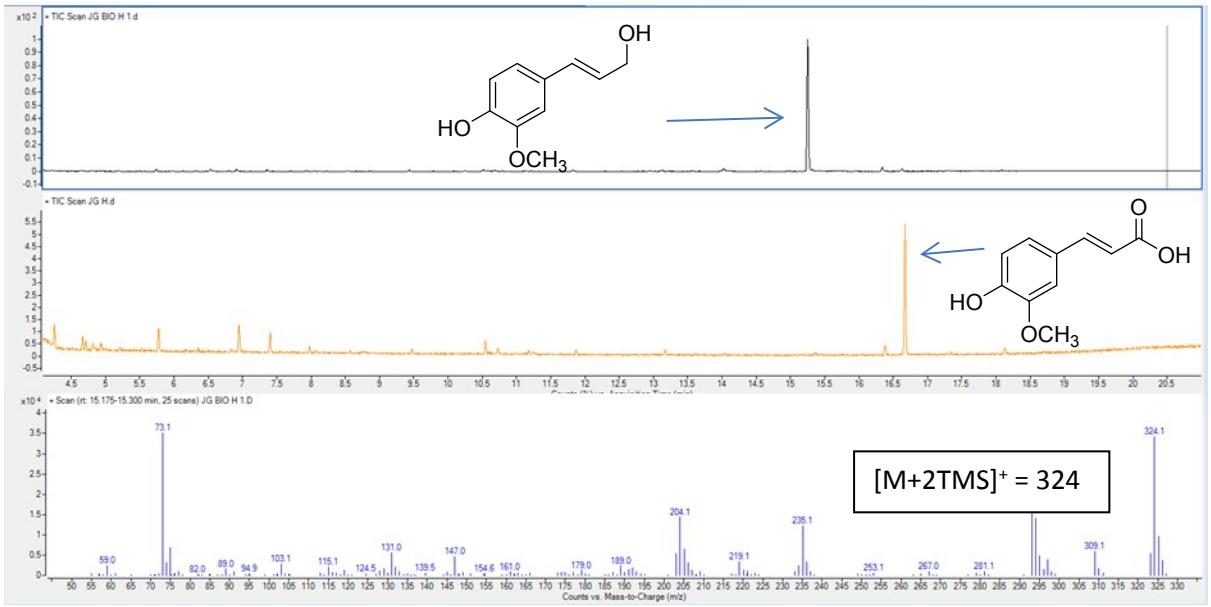


Purified coniferyl aldehyde **2**

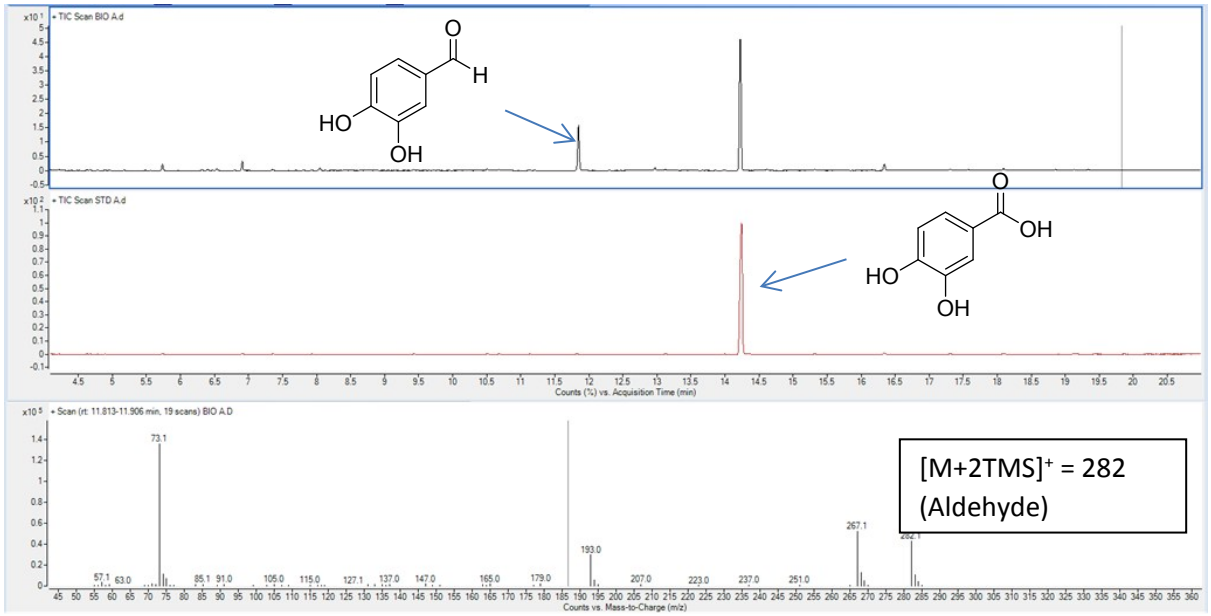


Supplementary Figure S5. High resolution mass spectroscopy analysis of purified coniferol 3 and coniferyl aldehyde 2 isolated following whole-cell biocatalytic production using the whole cell NiCAR-CgAKR-1 route. Coniferol **3** HRMS (ESI): *m/z* for $[M–H]^-$: $C_{10}H_{11}O_3^-$ calcd 179.0708, found 179.0720. Coniferyl aldehyde **2** HRMS (ESI): *m/z* for $[M+H]^+$: $C_{10}H_{11}O_3^+$ calcd 179.0708, found 179.0707.

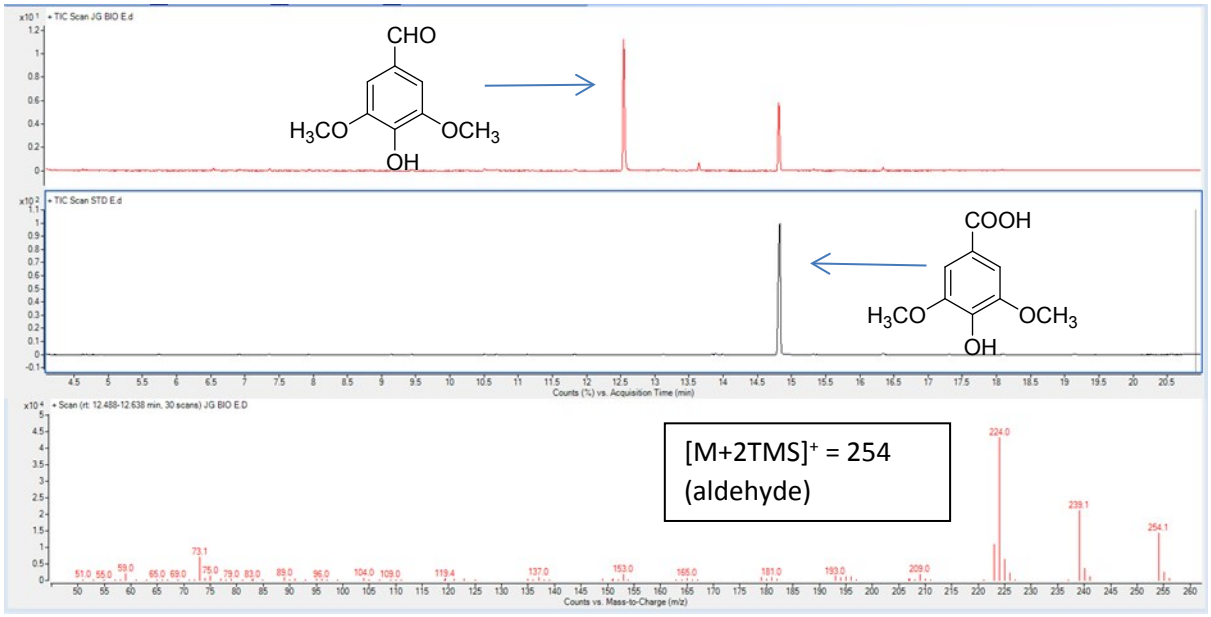
1) Ferulic acid (>99% alcohol)



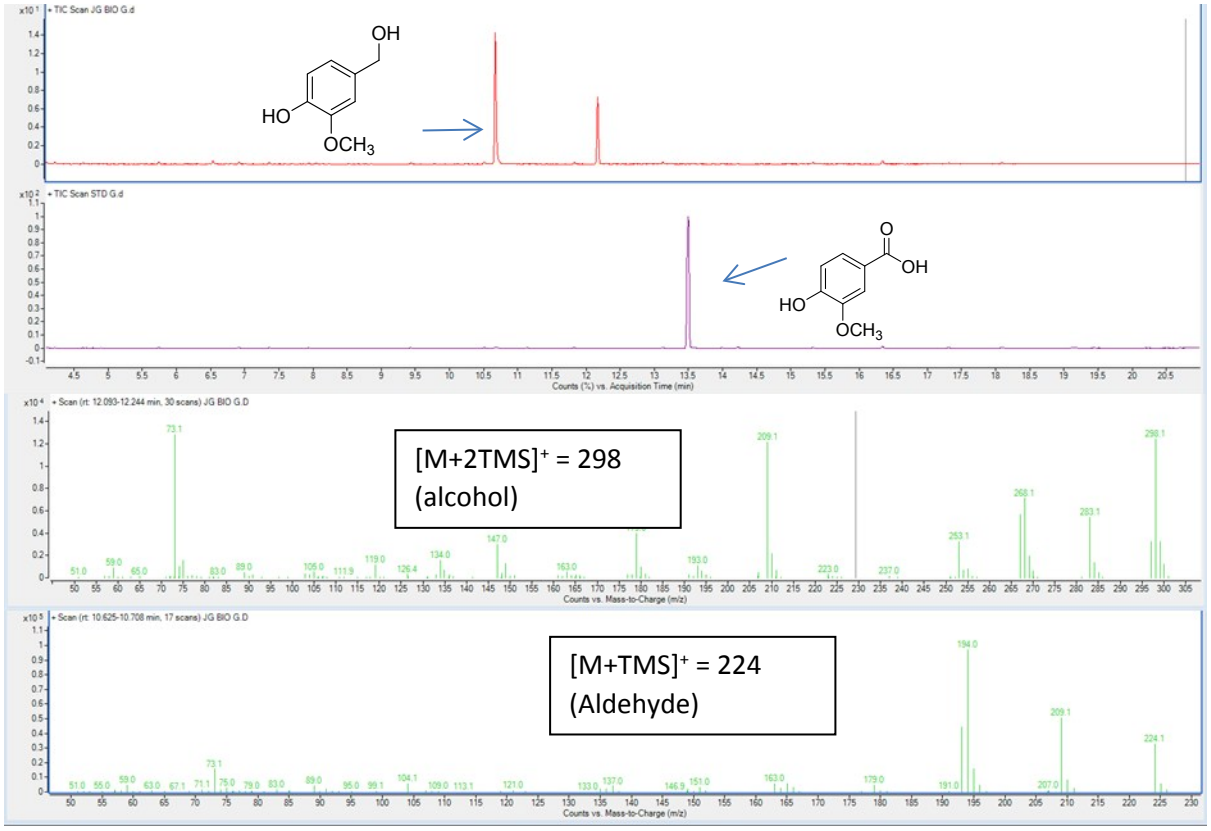
4) Protocatechuic acid (23% to aldehyde, 77% acid) No alcohol detected.



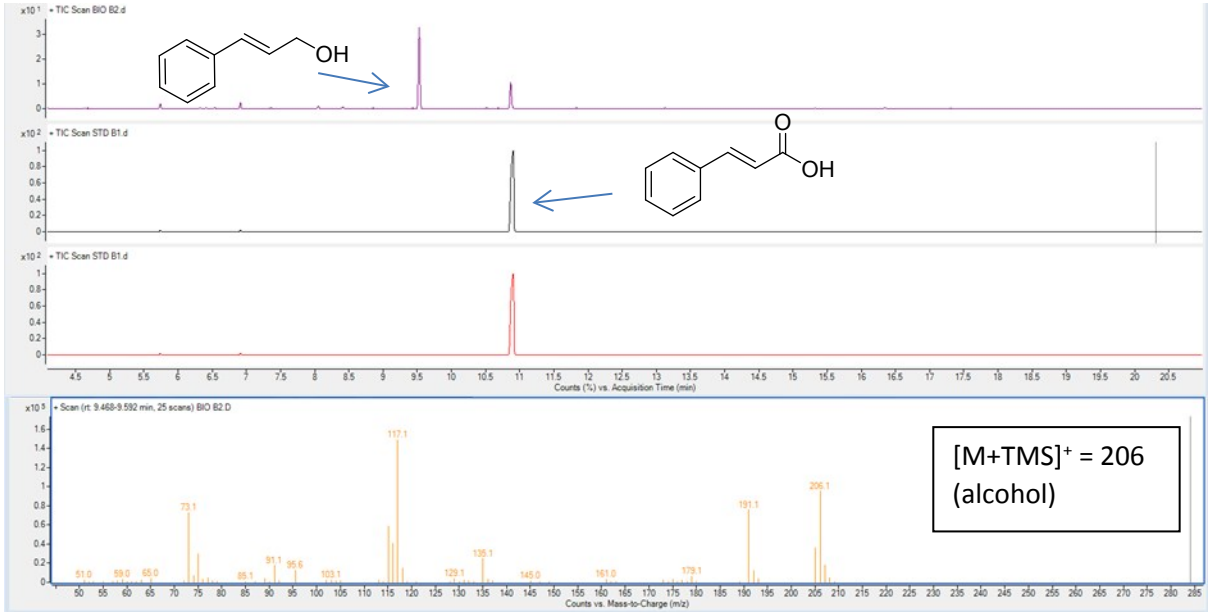
5) Syringic acid (68% aldehyde, 32% acid)



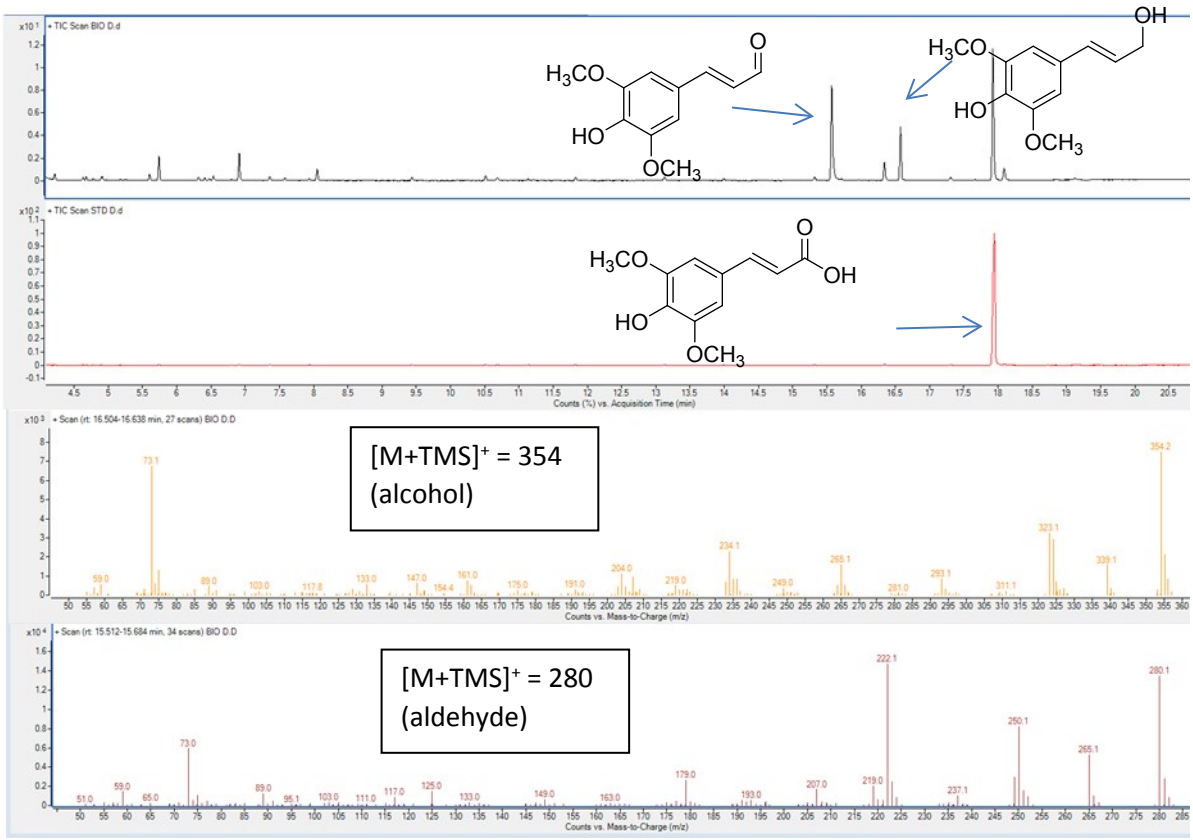
6) Vanillic acid (33% aldehyde, 67% alcohol)



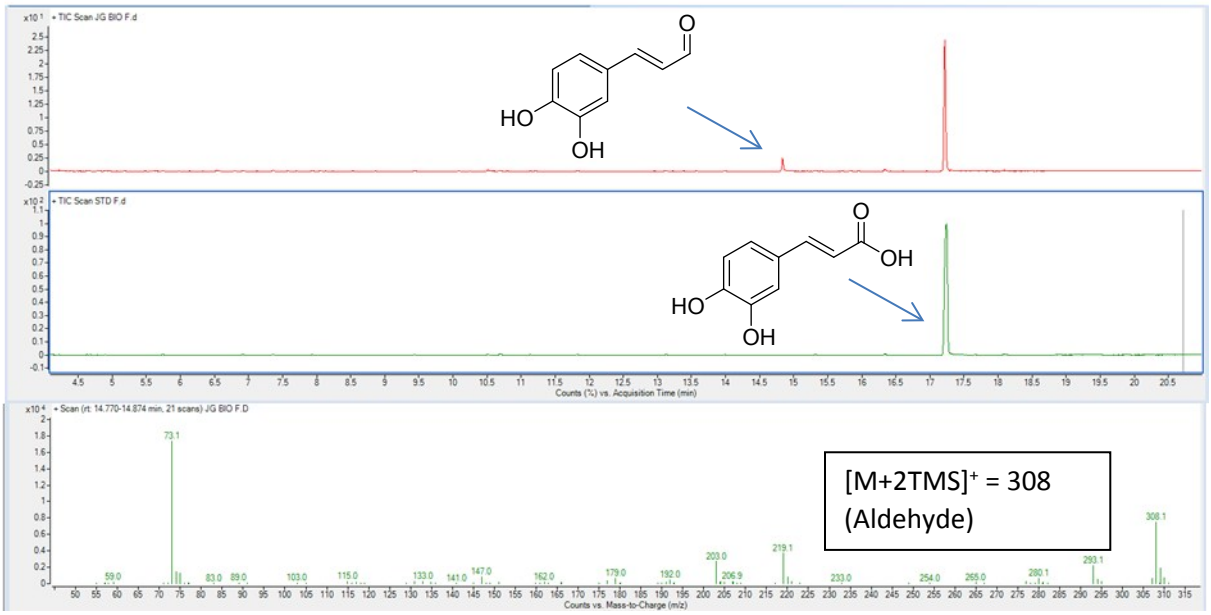
7) Cinnamic Acid (76% alcohol, 24% acid)



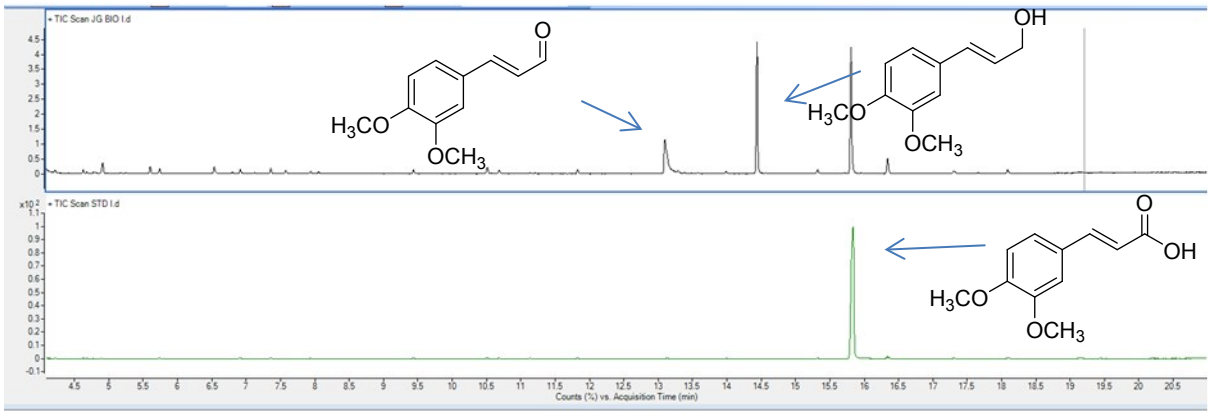
8) Sinapinic acid (36% aldehyde, 18% alcohol, 45% acid)

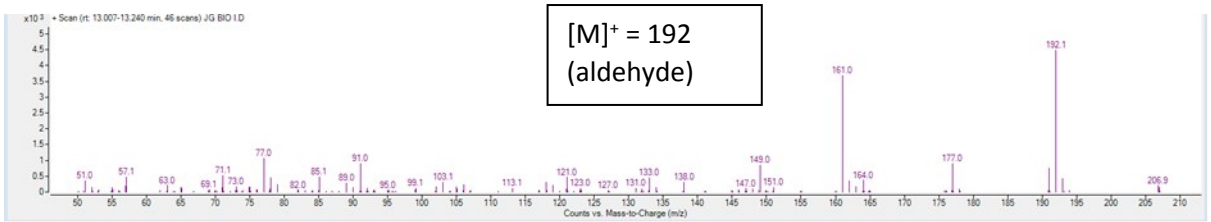


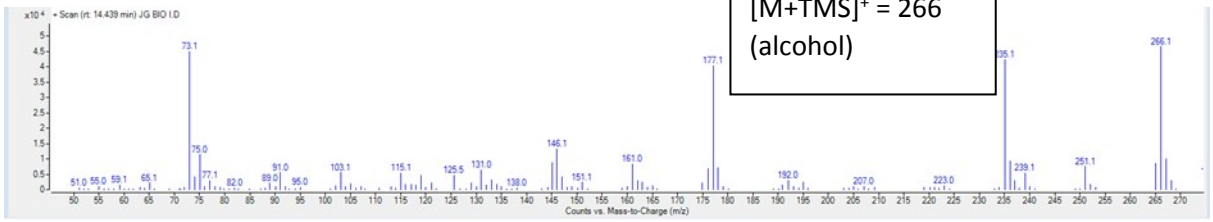
9) Caffeic acid (9% aldehyde, 91% acid)



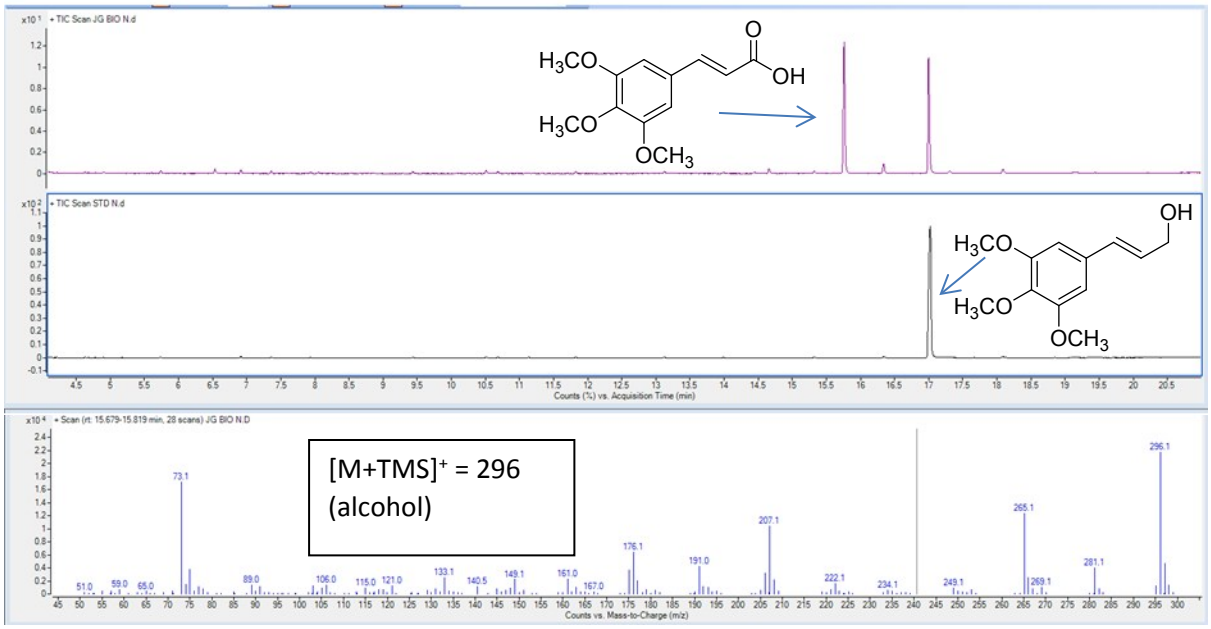
10) 3, 4 dimethoxy cinnamic acid (22% aldehyde, 37% alcohol, 40% acid)



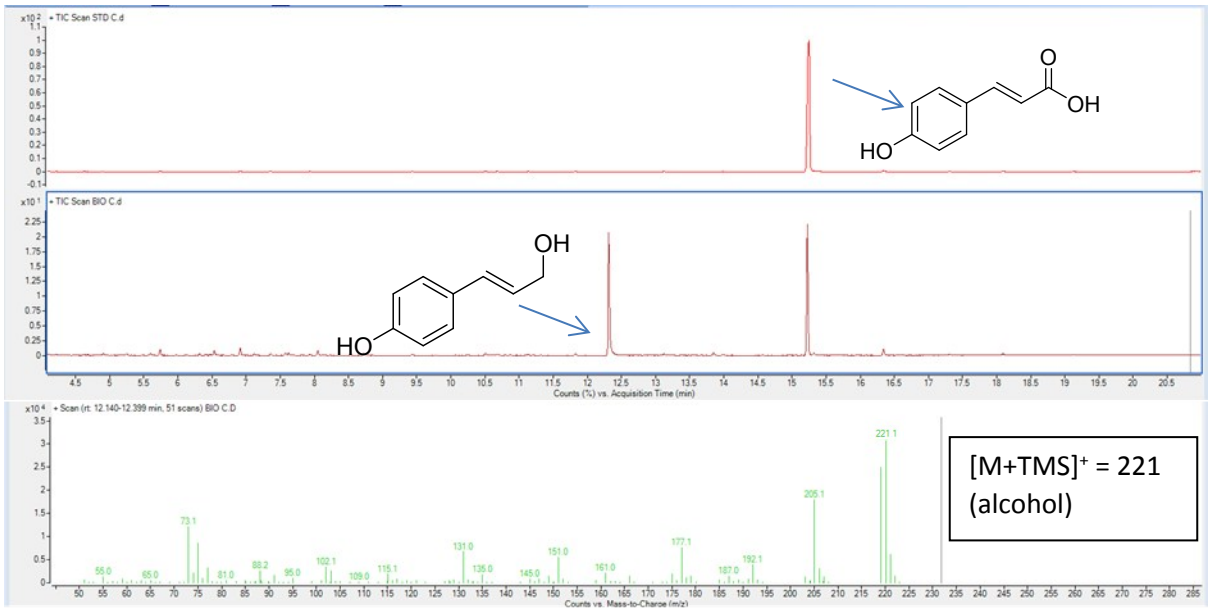




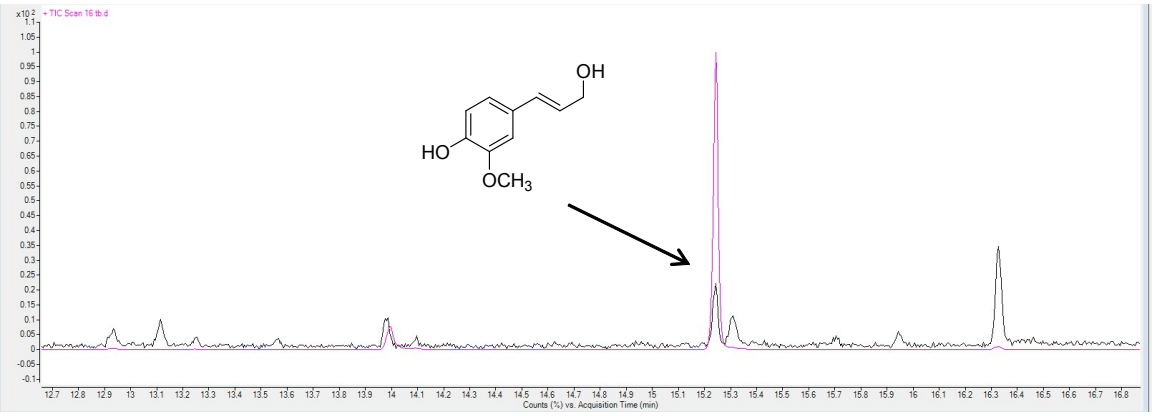
11) 3, 4, 5-trimethoxy cinnamic acid (52% aldehyde, 48% acid)



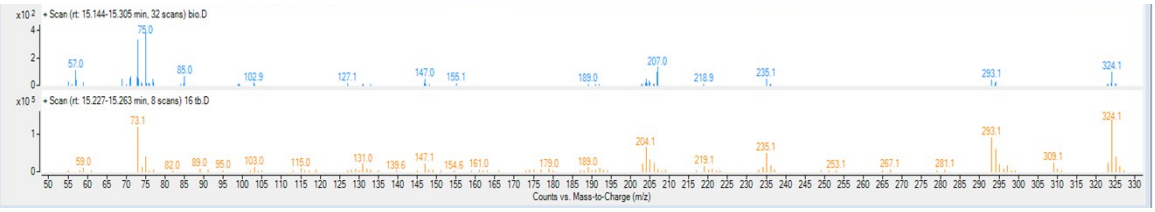
12) Coumaric acid (49% alcohol, 51% acid)



13) Overlay of WAX biotransformation (black) with coniferyl alcohol standard (pink)

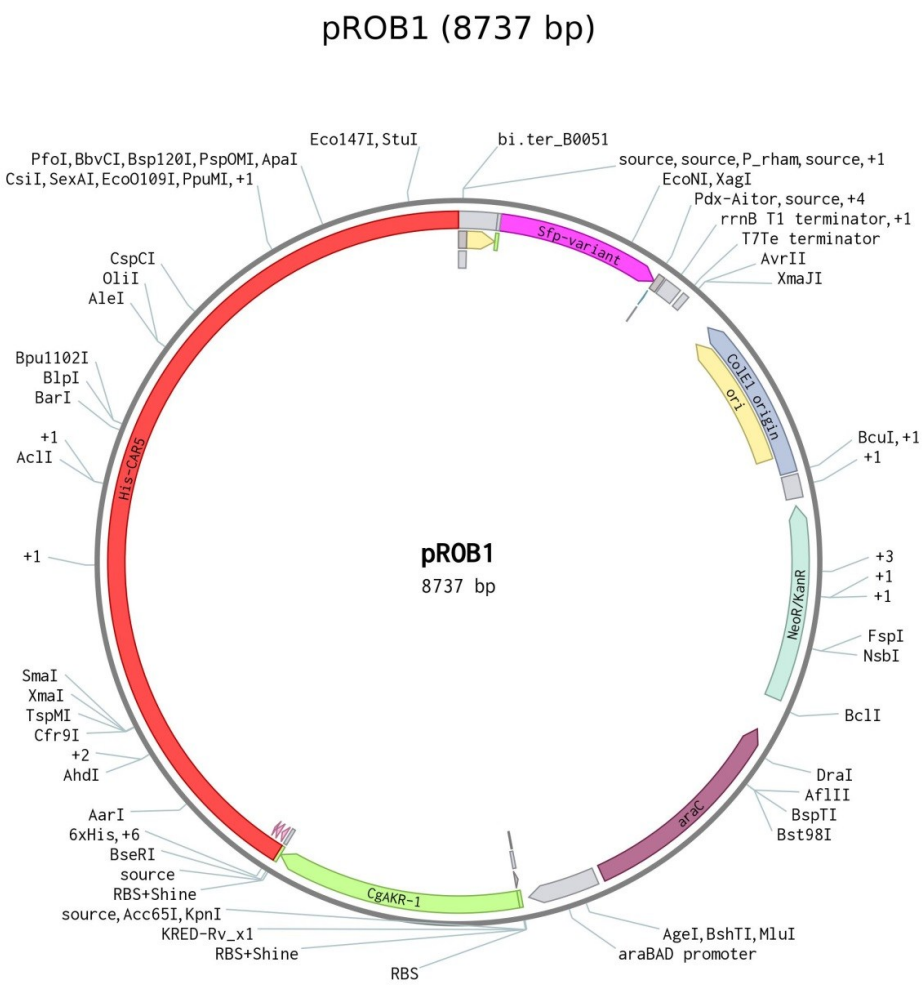


Mass spectra of WAX biotransformation (blue) and coniferyl alcohol standard (orange)



Supplementary Figure S6. GC-MS analysis of various substrates following whole-cell biocatalytic production using the whole cell NiCAR-CgAKR-1 route. For each compound the top panel shows the GC-MS chromatogram after whole cell biotransformation with NiCAR-CgAKR-1 route, the middle panel(s) is the GC-MS chromatogram of the standards, and bottom panel show the ionization spectrum and predicted ionization patterns of the main peaks from GC-MS chromatogram. For data summary see Fig. 6.

Plasmid construction information



Supplementary Figure S7. Schematic representation of the vector pROB1 used for biotransformations in this work.

Supplementary Table S2: Plasmid Construct Descriptions.

Num.	Name	Description	Function	Ref.
1	pET28B_XynZ	Naturally chimeric enzyme comprising activities of xylanase and feruloyl esterase (CE1) from *Clostridium thermocellum – XynZ* (#M22624.1). His-tag, IPTG inducible and kanamycin resistance.	Protein expression and enzymatic assays	4
2	pET28B_MCAR	*Mycobacterium marinum* CAR - MCAR (#WP_012393886.1.) His-tag, IPTG inducible and kanamycin resistance.	Protein expression and enzymatic assays	3
3	pET28B_TpCAR	*Tsukamurella paurometabola* CAR (WP_013126039.1). His-tag, IPTG inducible and kanamycin resistance.	Protein expression and enzymatic assays	5
4	pET28B_NiCAR	*Nocardia Iowensis* CAR – NiCAR (#Q6RKB1.1). His-tag, IPTG inducible and kanamycin resistance.	Protein expression and enzymatic assays	6
5	pET28B_SroCAR	*Segniliparus rotuduns* CAR - SroCAR (#WP_013138593.1). His-tag, IPTG inducible and kanamycin resistance.	Protein expression and enzymatic assays	7
6	pET28B_SrCAR	*Segniliparus rugosus* CAR – SrCAR (#E5XP76). His-tag, IPTG inducible and kanamycin resistance.	Protein expression and enzymatic assays	3,7,8
7	pET28B_CgAKR-1	*Coptotermes gestroi* aldo-keto reductase – *CgAKR-1* (#5KET_B). His-tag, IPTG inducible and kanamycin resistance.	Protein expression and enzymatic assays	9
8	pCDF1b_SFp	Phosphopantetheinyl transferase from *Bacillus subtilis - SFp* (#P39135). His-tag, IPTG inducible and streptomycin resistance CloDF13 as origin of replication.	Co-expression with CARs when in pet28B	3
9	pZZ-Eva2	Biobrick plasmid originated pBbE8Krfp containing PAL (Q3M5Z3) + SFp + KRED1 (WP_012427523.1) + SrCAR). Arabinose and Rhamnose inducible promoter, kanamycin resistance and ColE1 as the replication origin.	Biotransformation experiments	1,3
10	pZZ-Eva3	pZZeva2 originated (SFp + KRED1 + SrCAR).	Biotransformation experiments	This work
11	pZZ-Eva4	pZZeva3 originated (SFp + CgAKR-1 + SrCAR)	Biotransformation experiments	This work
12	pROB	pZZeva4 originated (SFp + CgAKR-1 + NiCAR)	Biotransformation experiments	This work
13	pZZ-Eva6	pZZeva4 originated (SFp + SrCAR)	Biotransformation experiments	This work
14	pZZ-Eva7	pZZeva5 originated (SFp + NiCAR)	Biotransformation experiments	This work

Supplementary Table S3. Basic information on oligonucleotides used for polymerase chain reactions (PCR) in this study.

Primer	sequence	Function
Eva2 FWD	CGTATGATGAGCCTTATTAGaggaggATAAAGAAATGGGCAGCAG	Open pZZ-Eva2 backbone to insert CgAKR-1
Eva2 REV	CTGCTCAGTTGTTTAGGCATTTCTTTATcctcctttaGTCCCGC	Open pZZ-Eva2 backbone to insert CgAKR-1
AKR FWD	GACtaaaggaggATAAAGAAATGCCTAAACAACTGAGCAG	CgAKR-1 amplification from CgAKR-1_pet28B
AKR REV	GCCCATTTCTTTATcctcctCTAATAAGGCTCATCATACGGG	CgAKR-1 amplification from CgAKR-1_pet28B
WPAL FWD	ATGCCTAAACAACTGAGCAG	Remove PAL from pZZ-Eva2
WPAL REV	TTCTTTATcctcctaGATCTTTTGAATTCC	Remove PAL from pZZ-Eva2
EVA2-AKR SEM PAL 2 FWD	AACTGCTTCAGCTGCTCTGAaaagtcaaaagcctccgacc	Replace SrCAR by NiCAR
EVA2-AKR SEM PAL 2 REV	TGATGATGGCTGCTGCCCATTTCTTTATcctcctCTAATAAGGC	Replace SrCAR by NiCAR
pET28b-NICAR_NheIBamHI FWD	TATTAGaggaggATAAAGAAATGGGCAGCAGCCATCATCA	Insert NiCAR amplification from pet28A
pET28b-NICAR_NheIBamHI REV	ggtcggaggcttttgactttTCAGAGCAGCTGAAGCAGTT	Insert NiCAR amplification from pet28A

Supplementary Table S4. Comparison of different biocatalytic routes and substrates to Coniferol

Pathway	Substrate metrics		Product metrics		Value added	Ref
TAL-4CL-CCR-CAD	**Substrate**	**Glycerol**	**Product**	**3**	7	[10]
	Unit Cost (€/kg): [11]	143	**Unit Value (€/g)** [12]	275.00		
	Used (g/L)	30.00	**Produced (g/L)**	0.124		
	Substrate Cost (€/L)	4.29	**Product Value (€/L)**	34.10		
	Media Costs (€/L) §	0.81	-	-		
4CL-CCR-CAD	**Substrate**	**1**	**Product**	**3**	33	[13]
	Unit Cost (€/g): [14]	5.05	**Unit Value (€/g)** [12]	275		
	Used (g/L)	0.49	**Produced (g/L)**	0.33		
	Substrate Cost (€/L)	2.47	**Product Value (€/L)**	90.75		
	Media Costs (€/L) §	0.31	-	-		
4CL-CCR-CAD	**Substrate**	**1**	**Product**	**3**	23	[15]
	Unit Cost (€/g): [14]	5.05	**Unit Value (€/g)** [12]	275		
	Used (g/L)	0.20	**Produced (g/L)**	0.108		
	Substrate Cost (€/L)	0.98	**Product Value (€/L)**	29.70		
	Media Costs (€/L) §	0.31	-	-		
CAR-AKR	**Substrate**	**1**	**Product**	**3**	47	this study
	Unit Cost (€/g [14]	5.05	**Unit Value (€/g)** [12]	275		
	Used (g/L)	0.97	**Produced (g/L)**	0.85		
	Substrate Cost (€/L)	4.90	**Product Value (€/L)**	233.75		
	Media Costs (€/L) §	0.09	-	-		
XynZ-CAR-AKR	**Substrate**	**WAX**	**Product**	**3**	1	this study
	Unit Cost (€/kg): : [16]	893.00	**Unit Value (€/g)** [12]	275		
	Used (g/L) *	20.00	**Produced (g/L)**	0.071		
	Substrate Cost (€/L)	17.860	**Product Value (€/L)**	19.53		
	Media Costs (€/L) §	0.09	-	-		
	Enzymes Costs (€/L) ¶	0.01	-	-		
XynZ-CAR-AKR	**Substrate**	**WS**	**Product**	**3**	74	this study
	Unit Cost (€/kg): [17]	0.07	**Unit Value (€/g)** [12]	275		
	Used (g/L) *	20.00	**Produced (g/L)**	0.026		
	Substrate Cost (€/L)	0.001	**Product Value (€/L)**	7.15		
	Media Costs (€/L) §	0.09	-	-		
	Enzymes Costs (€/L) ¶	0.01	-	-		

Footnote: TAL-4CL-CCR-CAD: Tyrosine ammonia lyase, 4-coumaroyl-CoA ligase, cinnamoyl-CoA reductase, and a cinnamyl alcohol dehydrogenase. 4CL-CCR-CAD: 4-coumaroyl-CoA ligase, a cinnamoyl-CoA reductase, and a cinnamyl alcohol dehydrogenase. CAR-AKR: carboxylic acid reductase (CAR) and aldo-keto reductase. XynZ-CAR-AKR: Endo-1,4-beta-xylanase Z (containing the feruloyl esterase domain), carboxylic acid reductase, and aldo-keto reductase.* based on units of biomass used per reaction volume. ** based on units of product used per reaction volume. Value added based only on the substrate and media/enzyme costs (Table S5) and does not consider process cost.

Supplementary Table S5. Media costs. The prices of all media components calculated using Chemical E-Commerce platform. [16]

This work			
Raw Material	**Price (€/kg)**	**Used/L of cultivation (g)**	**Price (€/L)**
Glycerol	1.04	4	0.004
Yeast extract	3.09	24	0.074
Tryptone	0.27	12	0.003
Kanamycin Sulfate	36.32	0.03	0.001
Rhammanose	2.07	1.5	0.003
Arabinose	0.58	1.64	0.001
Phosphate buffer	1.17	1.63	0.002
Total media/induction costs			**0.089**
Yuan [10] Modified M9 (M9Y)			
Yeast extract	3.09	5	0.015
Kanamycin Sulfate	36.32	0.03	0.001
Ampicilin	50	0.03	0.002
Glucose	0,66	2.5	0.002
IPTG	601	0.238	**0.143**
Phosphate buffer	1.17	1.5	0.002
MOPS	324	2	**0.65**
NH4Cl,	0.44	0.54	0.00
CaCl2	0.44	0.22	0.00
MgSO4	0.48	0.012	0.00
Total media/induction costs			**0.813**
Aschenbrenner [13] LB médium			
Raw Material	**Price (€/kg)**	**Used/L of cultivation (g)**	**Price (€/L)**
Yeast extract	3.09	5	0.015
Tryptone	0.27	10	0.003
Kanamycin Sulfate	36.32	0.03	0.001
IPTG	601	0.476	**0.286**
NaCl	0.1	10	0.001
Total media/induction costs			**0.306**
Jiang [15] LB medium			
Raw Material	**Price (€/kg)**	**Used/L of cultivation (g)**	**Price (€/L)**
Yeast extract	3.09	5	0.015
Tryptone	0.27	10	0.003
Kanamycin Sulfate	36.32	0.03	0.001
Ampicilin	50	0.03	0.002
IPTG	601	0.476	0.286
NaCl	0.1	10	0.001
Total media/induction costs			**0.308**

Supplementary Table S6A - numerical values in support of Table 1

	Ferulic acid		Coumaric acid		Coniferyl alcohol		Coumaryl alcohol	
	mg/L	std	mg/L	std	mg/L	std	mg/L	std
WS XynZ	26	2	18	1	0	0	0	0
WS NiCAR-CgAKR-1 XynZ	0	0	0	0	26	2	18	1
WS 2M NaOH	25	4	5	1	0	0	0	0
WAX XynZ	60	4	54	4	0	0	0	0
WAX NiCAR-CgAKR-1 XynZ	0	0	0	0	71	4	59	4
WAX 2M NaOH	54	6	9	2	0	0	0	0

Supplementary Table S6B - numerical values in support of Figure 3

		%						mg/L					
	Time (h)	**1 (%)**	**std**	**2(%)**	**std**	**3 (%)**	**std**	**1 (mg/L)**	**std**	**2 (mg/L)**	**std**	**3 (mg/L)**	**std**
	0	100	5	0	0	0	0	194	10	0	0	0	0
	0.3	93	4.75	7	0.05	0	0	181	9	12	0	0	0
A	0.5	88.6	3.6	11.4	1.2	0	0	172	7	20	2	0	0
	4.7	10	2.85	88	1.8	2	0	19	6	157	3	4	0
	9.3	0	2.3	98	2.55	1	0	0	4	175	5	2	0
	18	0	2.3	100	2.55	0	0	0	4	178	5	0	0
	Time (h)	**1 (%)**	**std**	**2(%)**	**std**	**3 (%)**	**std**	**1 (mg/L)**	**std**	**2 (mg/L)**	**std**	**3 (mg/L)**	**std**
	0	100	5	0	0	0	0	194	10	0	0	0	0
	0.3	50	4.75	30	0.05	20	0	97	9	53	0	36	0
B	0.5	34.7	3.6	37.3	1.2	28	0	67	7	66	2	50	0
	4.7	3	2.85	39	1.8	58	0	6	6	69	3	104	0
	9.3	0	2.3	22	2.55	78	0	0	4	39	5	140	0
	18	0	2.3	2.1	2.55	97.9	0	0	4	4	5	176	0
	Time (h)	**1 (%)**	**std**	**2(%)**	**std**	**3 (%)**	**std**	**1 (mg/L)**	**std**	**2 (mg/L)**	**std**	**3 (mg/L)**	**std**
	0	100	5	0	0	0	0	194	10	0	0	0	0
	0.3	98	4.75	2	0.05	0	0	190	9	4	0	0	0
C	0.5	96	3.6	4	1.2	0	0	186	7	7	2	0	0
	4.7	80	2.85	20	1.8	0	0	155	6	36	3	0	0
	9.3	52	2.3	47	2.55	0	0	101	4	84	5	0	0
	18	22	2.3	78	2.55	0	0	43	4	139	5	0	0
	Time (h)	**1 (%)**	**std**	**2(%)**	**std**	**3 (%)**	**std**	**1 (mg/L)**	**std**	**2 (mg/L)**	**std**	**3 (mg/L)**	**std**
	0	100	5	0	0	0	0	194	10	0	0	0	0
	0.3	96.1	4.75	4	0.05	0	0	187	9	7	0	0	0
D	0.5	90	3.6	10	3	0	0	175	7	18	5	0	0
	4.7	50	2.85	6	4	44	0	97	6	11	7	79	0
	9.3	27.9	2.3	10	5	62	0	54	4	18	9	112	0
	18	14	2.3	5	2.55	81	0	27	4	9	5	146	0

Supplementary Table S6C - numerical values in support of Figure 4

	Ferulic acid		Coniferyl aldehyde		Coniferyl alcohol	
	mg/L	std	mg/L	std	mg/L	std
SrCAR BL21	280	15	160	4	400	22
NiCAR BL21	330	15	44	18	496	45
SrCAR CgAKR-1 BL21	113	19	313	18	483	18
NiCAR CgAKR-1 BL21	22	45	202	15	690	15
SrCAR RARE	122	1	763	43	7	0
NiCAR RARE	188	1	620	43	100	35
SrCAR CgAKR-1 RARE	582	29	109	5	243	12
NiCAR CgAKR-1 RARE	321	29	272	5	318	12

Supplementary Table S6D - numerical values in support of Figure 5A

	1		2		3	
	mg/L	std	mg/L	std	mg/L	std
SrCAR BL21	280	15	160	4	400	22
NiCAR BL21	330	15	44	18	496	45
SrCAR CgAKR-1 BL21	113	19	313	18	483	18
NiCAR CgAKR-1 BL21	22	45	202	15	690	15
SrCAR RARE	122	1	763	43	7	0
NiCAR RARE	188	1	620	43	100	35
SrCAR CgAKR-1 RARE	582	29	109	5	243	12
NiCAR CgAKR-1 RARE	321	29	272	5	318	12

Supplementary Table S6E - numerical values in support of Figure 5B

	1		2		3	
Time (Minutes)	mg/L	std	mg/L	std	mg/L	std
0	971	68	0	0	0	0
30	863	67	0	4	78	6
60	788	44	0	5	113	6
90	400	28	1	10	400	28
120	153	12	0	2	741	58
240	80	3	0	0	810	45
480	59	4	0	2	810	57
600	0	0	0	5	850	64

Supplementary Table S6F - numerical value in support of Figure 6

	%						mg/L					
	Acid %		Aldehyde %		Alcohol %		Acid %		Aldehyde %		Alcohol %	
1	0	5	1	1	99	5	**0**	0	**3**	0	**278**	14
4	23	5	77	5	0	0	**75**	3.7	**275**	14	**0**	0
5	35	5	68	5	0	0	**88**	4.4	**185**	9	**0**	0
6	0	5	36	3	64	5	**0**	0.0	**117**	6	**210**	11
7	24	5	0	5	66	5	**81**	4.0	**0**	0	**250**	12
8	45	5	36	5	18	5	**100**	5.0	**86**	4	**43**	2
9	91	5	9	5	0	0	**253**	12.6	**27**	1	**0**	0
10	40	5	22	5	37	5	**96**	4.8	**57**	3	**96**	5
11	48	5	52	5	0	5	**101**	5.0	**116**	6	**0**	0
12	51	5	0	0	49	5	**155**	7.8	**0**	0	**165**	8

Supplementary Table S6G - numerical value in support of Figure 7

	Ferulic acid		Coumaric acid		Coniferyl alcohol		Coumaryl alcohol	
	mg/L	std	mg/L	std	mg/L	std	mg/L	std
WS XynZ	26	2	18	1	0	0	0	0
WS NiCAR-CgAKR-1 XynZ	0	0	0	0	26	2	18	1
WS 2M NaOH	25	4	5	1	0	0	0	0
WAX XynZ	60	4	54	4	0	0	0	0
WAX NiCAR-CgAKR-1 XynZ	0	0	0	0	71	4	59	4
WAX 2M NaOH	54	6	9	2	0	0	0	0

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