Supporting Information

The biocatalytic synthesis of syringaresinol from 2,6-dimethoxy-4-allylphenol in one-pot using a tailored oxidase/peroxidase system

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# Table of Contents

Experimental ........................................................................................................................................... S3

1 Chemicals and Reagents ........................................................................................................... S3

2 Site-directed mutagenesis of EUGO ......................................................................................... S3

3 Enzyme expression, purification and storage ........................................................................... S3

4 Enzyme characterization ........................................................................................................... S4

5 Biocatalytic synthesis of sinapyl alcohol .................................................................................. S4

6 Analysis of sinapyl alcohol formation using analytical HPLC................................................. S4

7 Biocatalytic synthesis of syringaresinol from 2,6-dimethoxy-4-allylphenol using EUGO I427A and HRP ................................................................................................................................................ S5

8 Analysis of syringaresinol formation using analytical HPLC and solid product using GPC and NMR .................................................................................................................................................. S5

9 One gram conversion of 2,6-dimethoxy-4-allylphenol using EUGO I427A and HRP ............ S5

Table S1 ............................................................................................................................................ S6

Table S2 ............................................................................................................................................ S6

Method S1 ........................................................................................................................................ S6

Figure S1 ........................................................................................................................................ S7

Figure S2 .......................................................................................................................................... S8

Figure S3 ......................................................................................................................................... S9

Figure S4 ......................................................................................................................................... S10

Figure S5 ......................................................................................................................................... S11

Figure S6 ......................................................................................................................................... S12

Figure S7 ......................................................................................................................................... S13
Experimental

1 Chemicals and Reagents
2,6-dimethoxy-4-allylphenol, sinapyl alcohol, DMSO-\textit{d6}, oligonucleotide primers, Tris, peroxidase from horseradish (HRP) were purchased from Sigma Aldrich (St Louis, MO, USA). All media components and ampicillin antibiotic were from Fischer Scientific chemicals (Pittsburgh, PA, USA). Solvents were purchased from JT Baker (Pittsburgh, PA, USA), Lab Scan Analytical Sciences (Gilwicze, Poland) and Macron Fine Chemicals (Center Valley, PA, USA). Pfu Ultra II Hotstart PCR Master Mix was purchased from Agilent (Santa Clara, CA, USA). The Qiaprep Spin Miniprep Kit was purchased from Qiagen (Hilden, Germany). DpnI was purchased from New England Biolabs (Ipswich, MA, United States).

2 Site-directed mutagenesis of EUGO
Mutagenesis was performed by using the Quickchange mutagenesis kit from Thermo Fischer Scientific (MA, USA). Ile427 was substituted for valine, alanine and glycine and Val166 was replaced by alanine using the primers shown in Table S1. The recipe and conditions for each of the three mutations are shown in Methods S1.

3 Enzyme expression, purification and storage
\textit{E.coli} NEB 10-\beta cells were grown in 5 mL Luria-Bertani (LB) medium at 37 °C overnight to saturation. The pre-cultures were then diluted 100 times in terrific broth (TB) medium and grown at 37 °C until the optical density measurement reached 0.6. Induction was performed by adding an amount corresponding to 0.02 \% (w/v) L-arabinose to the cells and transferring the culture to 30 °C for 18 h. Expression of each of the EUGO mutants and the wild type was done in 500 mL of culture in a 2 L flask. The pBAD-EUGO-His plasmid was provided by GECCO-Biotech (Groningen, The Netherlands). Cells were harvested by centrifugation at 6000 X \textit{g} (Beckman Coulter, Avanti JE centrifuge, JLA 14 rotor) for 20 min at 4 °C. The pellets containing a mutant or the wild type enzyme were washed with Buffer A. They were then disrupted by sonication (10 min total time with cycles of 10 s on and 10 s off at 70 \% amplitude) using a Sonics Vibra-Cell VCX130 probe sonicator (Newtown, CT, USA). The cell-free extract was obtained by centrifugation at 12,000 x \textit{g} for 45 min at 4 °C. The extracts were filtered using Whatman FP 30/0.45 CA-S membrane syringes (GE Healthcare Lifesciences, Uppsala, Sweden) to remove remaining cell debris.

EUGO wild type and mutant enzymes were purified by an ÄKTA purifier (GE Healthcare Lifesciences) using a 5 mL HisTrap HP column (GE Healthcare Lifesciences). The column was first equilibrated using Buffer A followed by loading of the cell-free extract. Buffer B was then used to wash off any non-specific proteins from the column. The enzymes were then eluted from the column by using a gradient of Buffer C starting from 0 to 500 mM imidazole within 30 min. After purification, the proteins were
desalted by using the HiPrep 26/10 Desalting column (GE Healthcare Lifesciences) using Buffer D. All enzymes were frozen using liquid nitrogen and stored at -20 °C until further use. Buffer compositions are mentioned in the Supporting information, Table S2.

4 Enzyme characterization

Wild type EUGO and mutant concentrations were determined based on the absorbance of the covalently bound flavin cofactor at 441 nm using the molar extinction coefficient of EUGO at 441 nm = 14.2 mM⁻¹cm⁻¹. HRP was added from a 65 µM stock solution in 20 mM potassium phosphate buffer (KPi), pH 7 unless otherwise stated. The concentration of HRP was determined based on its molecular weight (MW ≈ 44,000 Da). For determination of steady state kinetic parameters of wild type EUGO versus I427A, I427V, I427G and V166A mutants for 2,6-dimethoxy-4-allylphenol, the reaction was monitored by following the increase in absorbance at 270 nm due to product formation (ε = 14.1 mM⁻¹cm⁻¹ at pH 7.5) using 50 mM Tris HCl at 25 °C.

5 Biocatalytic synthesis of sinapyl alcohol

Sinapyl alcohol was synthesized in a 2 mL reaction volume in 15 mL screw-cap pyrex tubes containing 20 mM KPi pH 7, 0.5 and 5 µM EUGO I427A, 5 % (v/v) DMSO and 10 mM 2,6-dimethoxy-4-allylphenol. The experiment was conducted at 30 °C/50 rpm in an INNOVA 40 New Brunswick Incubator Shaker (Edison, NJ, USA). The experiment was also repeated using 500 nM of EUGO mutants.

6 Analysis of sinapyl alcohol formation using analytical HPLC

Samples from the synthesis of sinapyl alcohol using EUGO I427A were taken at various time intervals. The samples were centrifuged at 14,000 rpm for 10 min. The supernatant was then filtered using a Millex-FH, PTFE filter (0.45 µm pore size) (Kenilworth, NJ, USA) to remove remaining debris and then injected for analysis on a JASCO HPLC system (Easton, MD, USA). A 10 µL volume of the sample was injected into a Grace Alltima HP C18 column (3 µm, 2.1 x 100 mm, with a precolumn of the same material). As references, sinapyl alcohol and 2,6-dimethoxy-4-allylphenol (4 mM stock solutions) were also injected. The solvents used for the system were: a) water with 0.1 % v/v formic acid and b) acetonitrile with 0.08 % formic acid. The HPLC method was: 2 min 10 % B, 18 min on a gradient of 10-70 % B, 3 min 70 % B followed by a 6 s decreased gradient of 70 – 10 % B and re-equilibration for 7 min. Detection was done at a wavelength of 280 nm.
Biocatalytic synthesis of syringaresinol from 2,6-dimethoxy-4-allylphenol using EUGO I427A and HRP

Syringaresinol was synthesized from 2,6-dimethoxy-4-allylphenol using the same conditions as mentioned for the synthesis of sinapyl alcohol (see above) with the addition of an amount equivalent to 0.65 µM HRP to the reaction medium. The experiment was done using 5 µM EUGO I427A.

Analysis of syringaresinol formation using analytical HPLC and analysis of insoluble product using GPC and NMR

Syringaresinol production was analyzed using the same procedure as mentioned for the synthesis of sinapyl alcohol (see above). In addition to a soluble product, also some precipitate was formed which was isolated and analyzed after washing with water by GPC and NMR. For GPC analysis, a 10 mg/mL sample of the dried solid in THF (containing 1% toluene as internal standard) was prepared. Subsequently, the solution was filtered (0.2 µm syringe filter) and applied on a Hewlett Packard 1100 GPC system equipped with three PL-gel 3 µm MIXED-E columns in series, a GBC LC 1240 RI detector operated at 35 ºC, using THF (1 mL/min) as a mobile phase at 42 ºC, calibrated on known polystyrene standards. NMR analysis was performed using a DMSO-d6 on a Bruker Ascend 600.

One gram conversion of 2,6-dimethoxy-4-allylphenol using EUGO I427A and HRP

In a 2 L flask, 250 mL reaction volume containing KPi 20 mM pH 7, EUGO I427A 3 µM, HRP 0.65 µM, DMSO 5 % (v/v) and a volume equivalent to one gram 2,6-dimethoxy-4-allylphenol was added. The flask was placed at 30 ºC/ 50 rpm and samples were routinely taken to analyze when the substrate was fully consumed. The samples were analyzed by analytical HPLC as mentioned before. The solution was filtered using a pre-weighed 47 mm glass fiber filter from PALL (Ann Arbour, MI, USA) under vacuum. The reaction mixture was extracted three times with ethyl acetate (3 x 250 mL), and then the combined organic extracts were washed with brine, dried over anhydrous MgSO₄, filtered and concentrated by rotavap. The residue was purified by flash chromatography (SiO₂, dichloromethane/methanol = 99:1).
Table S1. Primers used for the quickchange mutagenesis of isoleucine at 427 position in EUGO.

| Mutation | Primer Direction | Primer Sequence                  |
|----------|------------------|----------------------------------|
| I427V    | Forward          | ACGCCGCGCAATTCGTGATCGGGCTCCGC    |
| I427V    | Reverse          | GCGGAGCCCGATACGAATTGCAGCGGCCT    |
| I427A    | Forward          | GCAGATCGGCTCCGAGATGCACCAC        |
| I427A    | Reverse          | GATTGCGAATTGCGGCGGTAGCCCTGTGATCC |
| I427G    | Forward          | TCAGATCGCAGCCGAGACCTCCGCAGATG    |
| I427G    | Reverse          | AGCCCGATGCCGAATTGCGGCGGTAGAG    |
| V166A    | Forward          | GGACCAGCGGCGGAGGATCACCC          |
| V166A    | Reverse          | GGGTGTAACCCCGGCGCCGCGGTCC       |

Table S2. Buffer compositions for the purification of wild type and mutant EUGO

| Buffer | Composition                                                        |
|--------|-------------------------------------------------------------------|
| A      | 50 mM potassium phosphate buffer [KPi], 0.5 M NaCl, 5% [v/v] glycerol, pH 8 |
| B      | 50 mM KPi, 0.5 M NaCl, 5% [v/v] glycerol, 5 mM imidazole, pH 8      |
| C      | 50 mM KPi, 0.5 M NaCl, 5% [v/v] glycerol, 500 mM imidazole, pH 8    |
| D      | 50 mM KPi, 150 mM NaCl, 10% [v/v] glycerol, pH 7.5                 |

Method S1. Recipe and conditions for the quickchange mutagenesis of isoleucine at position 427 into alanine, valine and glycine and valine at position 166 into alanine in EUGO

Recipe for the quickchange mutagenesis of isoleucine 427 of EUGO into alanine, valine and glycine and valine at position 166 into alanine

| Component                  | Quantity |
|----------------------------|----------|
| Pfu Ultra II Hotstart PCR Master Mix | 15 µL    |
| Primer Mix (10 µM)         | 2 µL     |
| pBAD-EUGO-Histag plasmid   | 5 – 50 ng|
| MilliQ H₂O                 | 12.6 µL  |
| Total volume               | 30 µL    |

PCR Program for I427V, I427A and V166A mutations

| Step                  | Temperature (°C) | Duration (min) |
|-----------------------|------------------|----------------|
| Initial Denaturation  | 95               | 4              |
| Amplification (33 cycles) | 67 (I427V) | 0.5            |
|                       | 57 (I427A)       | 0.5            |
|                       | 72 (V166A)       | 3              |
| Final Extension       | 72               | 15             |

PCR Program for I427G mutations

| Step                  | Temperature (°C) | Duration (min) |
|-----------------------|------------------|----------------|
| Initial Denaturation  | 95               | 4              |
| Amplification (5 cycles) | 62              | 0.5            |
|                       | 72               | 3              |
| Amplification (30 cycles) | 95              | 0.5            |
|                       | 52               | 0.5            |
|                       | 72               | 3              |
| Final Extension       | 72               | 15             |
Figure S1. The steady state kinetic curves for a) wild type EUGO, b) I427A EUGO, c) I427V EUGO, d) I427G EUGO and e) V166A EUGO using 2,6-dimethoxy-4-allylphenol as a substrate.
Figure S2. HPLC chromatograms showing complete depletion of 2,6-dimethoxy-4-allylphenol (Rt = 15.3 min) within 22 h to form sinapyl alcohol (Rt = 4.3 min) in a conversion containing 10 mM of the substrate and 5 µM of the EUGO I427A mutant. The chromatograms are labelled a) conversion after 2 h, b) conversion after 22 h, c) standard 1 mM 2,6-dimethoxy-4-allylphenol and d) standard 1 mM sinapyl alcohol.
Figure S3. HPLC chromatograms showing complete depletion of 2,6-dimethoxy-4-allylphenol (Rt = 15.3 min) and conversion to syringaresinol (Rt = 14 min) within 25 h upon adding EUGO I427A 5 µM and HRP 0.65 µM to 10 mM substrate. a) shows immediate formation of sinapyl alcohol (Rt = 4.3 min) and traces of syringaresinol upon addition of both enzymes to the reaction mixture. b) shows 3.5 h into the reaction c) shows 25 h into the reaction showing complete depletion of 2,6-dimethoxy-4-allylphenol with syringaresinol present as the major fraction (almost pure), d) standard 1 mM 2,6-dimethoxy-4-allylphenol and e) standard 1 mM sinapyl alcohol.
Figure S4. GPC analysis of the insoluble product obtained from the biocatalytic cascade reaction of 2,6-dimethoxy-4-allylphenol (1) with EUGO I427A and HRP.
Figure S5. 2D $^1$H $^{13}$C HSQC NMR analysis (600 MHz, DMSO-$d_6$) of the insoluble product (aided by 2D $^1$H $^{13}$C HMBC NMR analysis shown in Figure S6).
Figure S6. 2D $^1$H $^{13}$C HMBC NMR analysis (600 MHz, DMSO-$d_6$) of the insoluble product showing the correlations used for the identification of structural fragments shown in Figure S5.
Figure S7. $^1$H NMR and $^{13}$C NMR of (±)-syringaresinol purified from 1 g conversion of 2,6-dimethoxy-4-allylphenol using EUGO I427A and HRP

$^1$H NMR (400 MHz, CDCl$_3$) δ 6.58 (s, 4H, H-2, 6, 2', 6'), 5.53 (s, 2H, OH), 4.73 (d, $J$ = 3.9 Hz, 2H, H-7, 7'), 4.34 – 4.23 (m, 2H, m, H-9, 9'), 3.95 – 3.85 (m, 2H, H-9, 9'), 3.89 (s, 12H, OMe), 3.16 – 3.03 (m, 2H, H-8, 8').

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 147.1 (C-3, 5, 3', 5'), 134.2 (C-4, 4'), 132.0 (C-1, 1'), 102.6 (C-2, 6, 2', 6'), 86.0 (C-7, 7'), 71.8 (C-9, 9'), 56.3 (OMe), 54.3 (C-8, 8').