Supporting Information
Selective enzymatic transformation to aldehydes *in vivo* by fungal carboxylate reductase from *Neurospora crassa*

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**SUPPLEMENTARY INFORMATION**

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Vector map pETDuet1-EcPPTaseHTNcCAR

Figure S1. Illustration of the expression vector pETDuet1-EcPPTaseHTNcCAR. The plasmid encodes one T7 promoter in front of both expression cassettes, the T7 terminator behind the second expression cassette, an ampicillin resistance gene, the lacI gene for the two lac operators in front of every gene, a his tag at the 5’ end of the NcCAR and a pBR322-derived high copy origin of replication. The copy number is downregulated by the product of the rop gene and reaches a medium copy number. The two genes encoding EcPPTase and NcCAR were cloned in the two consecutive multiple cloning sites with Ncol and HindIII for EcPPTase and NdeI and Xhol for NcCAR.
2. Determination of kinetic parameters

**Figure S2** Saturation curve of the reduction of cinnamic acid 1a dissolved in DMSO; $K_m = 0.445 \pm 0.050$ [mM], $v_{\text{max,spec}} = 2.33 \pm 0.06$ [µmol min$^{-1}$ mg$^{-1}$].

**Figure S3** Saturation curve of the reduction of potassium piperonylate 2a dissolved in water; $K_m = 0.173 \pm 0.022$ [mM], $v_{\text{max,spec}} = 3.70 \pm 0.12$ [µmol min$^{-1}$ mg$^{-1}$].
3. Reaction monitoring by HPLC
   a. Determination of Cinnamic acid 1a reduction by HPLC/UV

   The analysis of 1a, 1b and 1c was carried out with a Kinetex 2.6µ Biphenyl 100A HPLC column (Phenomenex) with a Phenylhexyl Security Guard ULTRA cartridge (Phenomenex). The mobile phases were ammonium acetate (5 mM) and 0.5% v/v acetic acid in water and ACN at a flow-rate of 0.26 mL min⁻¹. A stepwise gradient was used: 25–55% ACN (5 min), 55–70% ACN (5.0–7.2 min) 70–90% ACN (7.2–7.5 min). After 90 s, the column was re-equilibrated to starting conditions. The compounds were detected at 254 nm (DAD). For 1a, 1b and 1c, calibration with authentic standards was done at 254 nm and linear interpolation used for their quantification.

   b. Effect of pyrophosphatase addition on cinnamic acid conversion

   Inhibition by pyrophosphate was studied by comparison of the NcCAR mediated conversion of cinnamic acid 1a with or without the addition of commercial inorganic pyrophosphatase from baker’s yeast (Sigma). NcCAR preparation after gel-filtration with a concentration of 0.35 mg mL⁻¹ was used for these experiments (0.0175 mg mL⁻¹ final concentration). The standard spectrophotometric assay was used and the time course of the reaction was monitored by analysis of duplicate samples that were taken after 30, 60, 90, 120 and 240 minutes. In parallel, the reactions were carried out in the presence of pyrophosphatase with a final concentration of 0.0175 mg mL⁻¹. The results are depicted in Figure S4.
Figure S4 Time course of cinnamic acid 1a reduction using NcCAR in vitro. Blue diamonds represent 1a concentrations, green triangles represent aldehyde 1b concentrations and red squares represent alcohol 1c concentrations. Top: without addition of inorganic pyrophosphatase; Bottom: with addition of inorganic pyrophosphatase.

c. Determination of Vanillic acid reduction by HPLC/MS

The analysis of vanillic acid (4-Hydroxy-3-methoxybenzoic acid), vanillin (4-hydroxy-3-methoxybenzaldehyde) and vanillyl alcohol (4-Hydroxy-3-methoxybenzyl alcohol) was carried out with a Kinetex 2.6µ Biphenyl 100A HPLC column (Phenomenex) with a Phenylhexyl Security Guard ULTRA cartridge (Phenomenex). The mobile phases were ammonium acetate (5 mM) and 0.5% v/v acetic acid in water and ACN at a flow-rate of 0.38 mL min⁻¹. A stepwise gradient was used: 0–20% ACN (5 min), 20–70% ACN (5.0–7.0 min) and 70–90% ACN (7.0–9.0 min). After 30 s, the column was re-equilibrated with aqueous phase. The compounds were detected at 254 nm (VWD) and negative scan mode (API-ES) as well as single ion monitoring of the acid (M-1 167), the aldehyde (M+1 153) and the alcohol (M+1 153).
4. Reaction monitoring and product confirmation by NMR
a. \(^1\text{H}\) and \(^{13}\text{C}\) NMR

When 1a reduction did not proceed any further as judged by \(^{31}\text{P}\) NMR, an \(^1\text{H}\) NMR was measured to assess the progress of cinnamic acid 1a reduction.

Figure S5 shows both cinnamic acid 1a and the respective aldehyde 1b in approximately 2:1 ratio. Piperonal was analyzed by \(^1\text{H}\) and \(^{13}\text{C}\) NMR, respectively (Figure S6 and Figure S7).

Figure S5 \(^1\text{H}\) NMR of NcCAR mediated reduction of cinnamic acid 1a after 4 h reaction time.
Figure S6. $^1$H NMR of piperonal 2b after NcCAR mediated reduction from piperonylic acid 2a, extraction into n-hexane and crystallization.

Figure S7 $^{13}$C NMR of piperonal 2b after NcCAR mediated reduction from piperonylic acid 2a, extraction into n-hexane and crystallization.