Supporting Information

Intrinsic Ability of the β-Oxidation Pathway To Produce Bioactive Styrylpyrones

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1. Supplementary Experimental Procedures

1.1 Chemicals and chemical analysis

All substrates and kavalactone standards used in this study are listed in Table S1. LC-HRMS was performed on a Q-Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer using electrospray ionization and a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific) equipped with a Kinetex C18 column (2.1 × 150 mm, 2.5 μm, 100 Å, Phenomenex). The analytical method was a gradient elution of solvents A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) at a flow rate of 0.3 mL/min: 5% B for 0.5 min, a linear gradient to 97% B for 11.5 min, then 97% B for 3 min, and 5% B for 3 min. Semi-preparative HPLC was performed on a Shimadzu LC-20AD instrument, equipped with a Kinetex C18 column (10 ×250 mm, 5 μm, 100 Å, Phenomenex) maintained at 30 °C. Purification was conducted using a linear gradient of solvents A (water, 0.1% formic acid) and B (acetonitrile) at a flow rate of 2 mL/min. NMR measurements were performed on a Bruker AVANCE III 600 MHz spectrometer, equipped with a Bruker Cryoplatform.

1.2 General molecular biology experiments

All PCR amplifications were performed using the Phusion Flash High-Fidelity DNA polymerase (Thermo Fisher Scientific). PCR products were purified with a Zymoclean™ Gel DNA Recovery Kit (Zymo Research Europe GmbH). Vector assembly was achieved by using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs GmbH).

1.3 Plasmid construction

All used primers are listed in the Table S2 while the plasmids used in this study are reported in the Table S3. The DNA sequence of 4CL from N. tabacum (UniProt: O24146)\(^1\) was synthesized by Thermo Fisher Scientific, amplified using primer pair [1]/[2], digested with BamHI and HindIII, and cloned into the pET28a_H6TEV expression vector\(^2\), in frame with an N-terminal 6His-tag and a cleavage site for the tobacco etch virus protease (TEV protease), resulting in the plasmid pET28a_4CL. The DNA sequence of BZL from R. palustris (UniProt: Q6NC13) was synthesized by ATG:biosynthetics GmbH, amplified using primer pair [3]/[4], digested with BamHI and HindIII, and cloned into the pET28a_H6TEV vector, obtaining the plasmid pET28a_BZL. The sequences of fadD, fadK, fadA, fadl, atoB, yqeF were amplified from E.coli DH5α genomic DNA using primers [5]/[6], [7]/[8], [9]/[10], [11]/[12], [13]/[14] and [15]/[16], respectively, and cloned into the BamHI/HindIII-linearized pET28a_H6TEV vector, to give the plasmids pET28a_FadD, pET28a_FadK, pET28a_FadA, pET28a_Fadl, pET28a_AtoB and pET28a_YqeF. The matA gene was isolated from R. trifolii (Jena Microbial Research Collection, JMRC:ST:036047) using primers [17]/[18], cloned into pJET 1.2 plasmid (Thermo Fisher Scientific), and sequenced. Afterwards, the matA-gene was amplified using primers [19]/[20], digested with BamHI and HindIII, and cloned into the pET28a_H6TEV vector, resulting in the plasmid pET28a_matA. The matB gene was isolated from the same organism using primers [21]/[22], cloned into pJET 1.2, and sequenced. Afterwards, the matB-gene was amplified using primers [23]/[24], digested with BamHI and HindIII, and cloned into the pET28a_H6TEV expression vector, resulting in the plasmid pET28a_matB. To reconstitute the α-pyrones pathway in E. coli, the gene sequences
of 4CL, BZL, fadD, fadK, fadA, fadI, KOMT1 (from *P. methysticum* (UniProt: A0A4Y5QN8) was synthesized by Biomatik) were amplified using primer pairs [25]/[26], [27]/[28], [29]/[30], [31]/[32], [33]/[34], [35]/[36] and [37]/[38], respectively, and inserted into the EcoRV-linearized pMGE-T7 vector[3], forming the plasmids pMGE_4CL, pMGE_BZL, pMGE_FadD, pMGE_FadK, pMGE_FadA, pMGE_FadI and pMGE_KOMT1. To combine the genes, the Swal-restricted pMGE_FadD plasmid was fused with the *Pmel/HindIII*-restricted pMGE_FadA or pMGE_FadI by Gibson assembly, resulting in plasmids pMGE_FadDA and pMGE_FadDI, respectively. The Swal-restricted pMGE_FadK plasmid in the same way to form plasmids pMGE_FadKA and pMGE_FadKI. The Swal-restricted pMGE_FadA and pMGE_FadI were individually fused with the *Pmel-*restricted pMGE_4CL and pMGE_BZL to yield pMGE_FadA_4CL, pMGE_FadA_BZL, pMGE_FadI_4CL and pMGE_FadI_BZL, respectively. To produce kavalactones *in vivo*, the tailoring enzyme O-methyltransferase KOMT1 was included in the plasmids pMGE_FadDA_KOMT1 and pMGE_FadDI_KOMT1 by assembling the *Pmel/HindIII*-restricted pMGE_KOMT1 with the Swal-restricted pMGE_FadDA and pMGE_FadDI, separately.

### 1.4 Strain construction

All strains used in this work are listed in the Table S4. The above mentioned pMGE plasmids containing different combinations of enzymes were used to transform *E. coli* BL21 (DE3) for *in vivo* production of α-pyrones. The *E. coli* single gene knockout mutants were purchased from Horizon Discovery Biosciences LTD.

### 1.5 Protein expression and purification

For expression of the ten enzymes 4CL, BZL, FadD, FadK, FadA, FadI, AtoB, YqeF, MatA and MatB, *E. coli* BL21 (DE3) cells containing the pET28a plasmids of corresponding enzymes were used. A pre-culture with LB-medium and 25 µg/mL kanamycin was inoculated with the corresponding cells and grown overnight at 37 °C while shaking. A main culture containing auto-induction medium[4] and 25 µg/mL kanamycin was inoculated with pre-culture in a 1:100 ratio using baffled shaker flasks. The main culture was incubated at 37 °C while shaking until OD600 reached approximately 1.0, then temperature was lowered to 18 °C for overnight incubation. Cells were harvested *via* centrifugation and stored at -20 °C. Then, cells were thawed, re-suspended in buffer A (0.1 M TRIS, 0.5 M NaCl, pH 8.0) and lysed by sonication (Sonopuls 2070, Bandelin, cycle 6, 75% intensity, 2 x 2 min) on ice. After centrifugation (16,000 x g, 4 °C, 20 min), supernatants were applied to a HisTrap FF crude column connected to an Aekta Explorer system (both GE Healthcare). After washing with 25 mM imidazole the proteins (4CL, BZL, FadD, FadK, FadA, FadI, AtoB, YqeF, MatA) were eluted from the column with 500 mM imidazole using buffer B (0.1 M TRIS, 0.5 M NaCl, 0.5 M imidazole, pH 8.0), while MatB was eluted with 125 mM imidazole. Protein containing fractions were analysed using Coomassie stained SDS-Page, pooled and protein concentrations were determined using Bradford assay.
1.6 In vitro production of α-pyrones

The enzymatic reactions shown in Figure 2 A were performed as follow: samples of 350 µL containing ATP (0.5 mM), acetyl-CoA (0.5 mM), each starting substrate (0.5 mM, 0.1 mM for acetoacetyl-CoA), HEPES pH 7.5 (20 mM), TCEP (0.5 mM), MgCl₂ (2.5 mM), ligase (0.2 µg/mL) and thiolase (10 µg/mL) were incubated at 37°C overnight. Afterwards, samples were extracted with equal volume of methanol and filtered for LC-HRMS analysis. Enzymatic reactions in Figure 3 were carried out in 150 µL containing ATP (0.5 mM), acetyl-CoA (0.5 mM), each starting substrate (0.5 mM), HEPES pH 7.5 (20 mM), TCEP (0.5 mM), MgCl₂ (2.5 mM), ligase (2.5 µg/mL, 0.25 µg/mL for BZL) and thiolase (10 µg/mL) were incubated at 37°C overnight. Then, samples were extracted with equal volume of methanol and filtered for LC-HRMS analysis. All samples were measured including at least three biological and three technical replicas.

1.7 Labelling experiment

For labelling experiment in Figure 2 B, samples of 350 µL containing ¹³C malonate (0.5 mM), ATP (0.5 mM), with or without acetoacetyl-CoA (0.5 mM), CoA (0.1 mM), HEPES pH 7.5 (20 mM), TCEP (0.5 mM), MgCl₂ (2.5 mM), MatA (0.2 µg/mL), MatB (2 µg/mL) and thiolase (10 µg/mL, control without thiolase) were incubated at 37°C overnight. Afterwards, samples were extracted with equal volume of methanol and filtered for LC-HRMS analysis.

1.8 PPᵢ-Assay for determining the substrate specificity of ligases

Standard curve of PPᵢ for molybdate-based activity assay was previously established[^3]. As shown in Figure 3, to analyse the substrate specificity of ligases (4CL, BZL, FadD and FadK), 100 µL of samples containing each substrate (0.5 mM), ATP (0.5 mM), CoA (0.5 mM), HEPES pH 7.5 (20 mM), TCEP (0.5 mM), MgCl₂ (2.5 mM), and each ligase (2 µg, 0.2 µg for 4CL) were incubated at 37°C (reactions of 4CL at 47°C) for 20 min. Blank samples contained all chemicals except CoA, being replaced by water. Enzymatic reactions were stopped by adding ammonium molybdate. For the detection of PPᵢ, all solutions were freshly prepared and stored on ice. The assay was performed on the basis of what was reported before[^3, 5]. All samples were measured including three biological and three technical replicas.

1.9 In vivo production of α-pyrones

For in vivo production of α-pyrones (Figure 4 A and 4 B; Figure 5 B), E. coli BL21 (DE3) was transformed with pMGE-T7 plasmids harbouring different enzymes (control strain contained empty pMGE-T7 plasmid) and grown in LB medium containing kanamycin (25 µg/mL) overnight (14-16 h) at 37°C and 180 rpm. Then, the overnight cultures were inoculated to fresh LB medium (with 25 µg/mL kanamycin) at starting OD₆₀₀ of 0.1 and incubated at 37°C while shaking until OD₆₀₀ of 1.0. Afterwards, the temperature was reduced to 18°C and the cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Next, the OD₆₀₀ of the cultures were measured. Bacteria were harvested by centrifugation for 5 min at 3220g at 4 °C and re-suspended with OD₆₀₀ of 2.0 in M9 medium containing kanamycin, 1 mM IPTG, 2% glucose, and 3 mM of corresponding substrate. Fermentation continued at 30 °C, shaking for 48 h. Pre-culture and main culture before changing to M9 medium were performed in 100 mL Erlenmeyer flasks filled with 20 and 30
mL of LB medium, respectively. After replacing the medium to M9, fermentations were conducted in 50 mL falcon tubes (the caps were slightly loosened) filled with 4 mL culture with OD$_{600}$ of 2.0. For metabolic analyses, cultures were extracted with 25 mL ethyl acetate. The organic phase was evaporated under reduced pressure, then the crude extract was dissolved in 300 μL methanol, filtered through a 0.2 μm PTFE filter (Carl Roth GmbH & Co. KG) and used for LC-HRMS measurement.

1.10 Production of α-pyrene 32a in different bacterial species

We tested the potential of different bacterial species kept in the laboratory to produce α-pyrene 32a when 32 was fed to the culture, and found that they produced 32a in different conditions. Fermentations were all performed in 100 mL Erlenmeyer flasks filled with 20-30 mL of medium. *P. fluorescens* was cultivated overnight (14-16 h) in LB medium at 30 °C, and used as the initial inoculum in fresh 30 mL LB medium at OD$_{600}$ of 0.1. Afterwards, the cultures were incubated at the same temperature until OD$_{600}$ reached approximately 1.0. Then, bacteria were harvested by centrifugation for 5 min at 3220g at 4 °C and re-suspended in 20 mL M9 medium containing 2% glucose, 1 mM IPTG and 3 mM 32. Flasks were further incubated at the same temperature for 48 h. *S. hawaiensis* and *S. griseus* were cultivated 2 days in 30 mL LB medium at 28°C and then directly harvested by centrifugation for 5 min at 3220g at 4 °C and re-suspended in 20 mL M9 medium containing 2% glucose, 1 mM IPTG and 3 mM 32. Afterwards, the cultures were incubated at 28°C for 4 days. *E. faecium* and *S. aureus* were cultivated in 30 LB medium at 37°C for 2 days and overnight (14-16 h), respectively. Then, the mediums were changed to 20 mL auto-induction medium[4] and incubated at 37°C for 4 days. *S. rapamycinicus* was cultivated in 30 mL LB medium at 28°C for 7 days due to the low growth. Then, the cells were harvested by centrifugation and re-suspended in 20 mL M9 medium containing 0.5% glycerol, 0.05% glucose, 0.2% α-lactose, 1 mM IPTG and 3 mM 32. Next, the culture was incubated for another 7 days. For metabolic analyses, cultures were extracted with 25 mL ethyl acetate (and destroy the cells in ultrasonic bath). The organic phase was evaporated under reduced pressure, then the crude extract was dissolved in 1 mL methanol, filtered, and used for LC-HRMS measurement.

1.11 Fermentation and purification of DDK

*E. coli* containing pMGE_FadDI_KOMT1 was used for the production of DDK. *E. coli* BL21 (DE) was freshly transformed with pMGE_FadDI_KOMT1 and cultivated overnight in a 300 mL Erlenmeyer flask filled with 100 mL LB medium (containing 25 μg/mL kanamycin). The main culture containing 300 mL LB medium (with 25 μg/mL kanamycin) in 1 L Erlenmeyer flask (300 mL x 6) was inoculated with pre-culture at starting OD$_{600}$ of 0.1 and incubated at 37°C while shaking until OD$_{600}$ of 1.0. Afterwards, the temperature was reduced to 18°C and the cultures were induced with 1 mM IPTG for 3 h. Cells were then harvested by centrifugation and re-suspended in M9 medium (20 mL x 60) containing 25 μg/mL kanamycin, 1 mM IPTG, 2% glucose, and 3 mM of 32. Fermentation continued at 30 °C, shaking for 48 h. After fermentation, the culture was extracted with the same volume of ethyl acetate three times. Then, the organic phase was evaporated under reduced pressure. The residue (615 mg) was purified twice using a semi-preparative HPLC (Shimadzu) equipped with a Kinetex C18 column (250 × 10 mm, 5 μm, 100 Å, Phenomenex, 2 mL/min, method: 0-3.0
min: 10%-40%; 3.0-23.0 min: 40%-63.5% (v/v) MeCN/ H2O containing 0.1% formic acid) to give DDK ($t_R$ 21.0 min, 0.7 mg).
2. Supplementary Tables

Table S1. Chemicals used in this study

| No. | Name                                           | CAS number  | Source                      |
|-----|------------------------------------------------|-------------|-----------------------------|
| 1   | cinnamic-acid                                  | 140-10-3    | Sigma-Aldrich               |
| 2   | 2-hydroxycinnamic acid                         | 614-60-8    | Sigma-Aldrich               |
| 3   | 2-methoxycinnamic acid                         | 1011-54-7   | Fisher Scientific GmbH      |
| 4   | 2-aminocinnamic acid                           | 1664-63-7   | Chemical synthesis[3, 6]     |
| 5   | 2-nitrocinamic acid                            | 612-41-9    | Th. Geyer GmbH & Co. KG     |
| 6   | 2-fluorocinnamic acid                          | 451-69-4    | Th. Geyer GmbH & Co. KG     |
| 7   | 2-chlorocinnamic acid                          | 3752-25-8   | TCI Deutschland GmbH        |
| 8   | 2-bromocinnamic acid                           | 7499-56-1   | TCI Deutschland GmbH        |
| 9   | 3-hydroxycinnamic acid                         | 14755-02-3  | Sigma-Aldrich               |
| 10  | 3-methoxycinnamic acid                         | 6099-04-3   | Fisher Scientific GmbH      |
| 11  | 3-aminocinnamic acid                           | 127791-53-1 | TCI Deutschland GmbH        |
| 12  | 3-nitrocinamic acid                            | 555-68-0    | Th. Geyer GmbH & Co. KG     |
| 13  | 3-fluorocinnamic acid                          | 20595-30-6  | Th. Geyer GmbH & Co. KG     |
| 14  | 3-chlorocinnamic acid                          | 1866-38-2   | Fisher Scientific GmbH      |
| 15  | 3-bromocinnamic acid                           | 7345-79-1   | TCI Deutschland GmbH        |
| 16  | p-coumaric acid                                | 501-98-4    | Sigma-Aldrich               |
| 17  | 4-methoxycinnamic acid                         | 830-09-1    | Sigma-Aldrich               |
| 18  | 4-aminocinnamic acid                           | 17570-30-8  | TCI Deutschland GmbH        |
| 19  | 4-nitrocinamic acid                            | 619-89-6    | Sigma-Aldrich               |
| 20  | 4-fluorocinnamic acid                          | 459-32-5    | Th. Geyer GmbH & Co. KG     |
| 21  | 4-chlorocinnamic acid                          | 1615-02-7   | Sigma-Aldrich               |
| 22  | 4-(trifluoromethyl)cinnamic acid               | 16642-92-5  | Sigma-Aldrich               |
| 23  | caffeic acid                                   | 331-39-5    | Sigma-Aldrich               |
| 24  | ferulic acid                                   | 537-98-4    | Carl Roth GmbH & Co. KG     |
| 25  | 3,4-dimethoxycinnamic acid                     | 2316-26-9   | Sigma-Aldrich               |
| 26  | umbellic acid                                  | 614-86-8    | Sigma-Aldrich               |
| 27  | 3,5-dihydroxycinnamic acid                     | 28374-93-8  | Chemical synthesis[3, 7]    |
| 28  | 2,3-(methyleneoxy)cinnamic acid                | 287118-44-9 | Chemical synthesis[3, 8]    |
| 29  | 3,4-(methyleneoxy)cinnamic acid                | 2373-80-0   | Sigma-Aldrich               |
| 30  | 3,4,5-trihydroxycinnamic acid                  | 6093-59-0   | Chemical synthesis[3, 7]    |
| 31  | sinapic acid                                   | 530-59-6    | Sigma-Aldrich               |
| 32  | 3-phenylpropionic acid                         | 501-52-0    | TCI Deutschland GmbH        |
| 33  | 3-(2-nitrophenyl)propionic acid                | 2001-32-3   | Activate Scientific GmbH    |
| 34  | 3-(2-chlorophenyl)propionic acid               | 1643-28-3   | Th. Geyer GmbH & Co. KG     |
| 35  | 3-(3-nitrophenyl)propionic acid                | 1664-57-9   | VWR International GmbH      |
| 36  | 3-(3-chlorophenyl)propionic acid               | 21640-48-2  | Th. Geyer GmbH & Co. KG     |
| 37  | 3-(4-hydroxyphenyl)propionic acid              | 501-97-3    | Th. Geyer GmbH & Co. KG     |
| 38  | 3-(4-methoxyphenyl)propionic acid              | 1929-29-9   | VWR International GmbH      |
| 39  | 3-(4-nitrophenyl)propionic acid                | 16642-79-8  | VWR International GmbH      |
| 40  | 3-(4-chlorophenyl)propionic acid               | 2019-34-3   | Th. Geyer GmbH & Co. KG     |
| 41  | 3-(4-(trifluoromethyl)phenyl)propionic acid    | 53473-36-2  | Th. Geyer GmbH & Co. KG     |
| 42  | 3-(3,4-dimethoxycinnamic)                     | 2107-70-2   | Th. Geyer GmbH & Co. KG     |
| 43  | 3-(3,4-methylenedioxyphenyl)propionic acid     | 2815-95-4   | Th. Geyer GmbH & Co. KG     |
| 44  | 3-(4-hydroxy-3,5-dimethoxyphenyl)propionic acid| 14897-78-0  | Activate Scientific GmbH    |
| 45  | phenylacetic acid                              | 103-82-2    | Th. Geyer GmbH & Co. KG     |
| 46  | phenylbutyric acid                             | 1821-12-1   | Th. Geyer GmbH & Co. KG     |
| 47  | phenylvaleric acid                             | 20620-59-1  | Th. Geyer GmbH & Co. KG     |
| 48  | phenoxyacetic acid                             | 122-59-8    | Sigma-Aldrich               |
| 49  | 3-(2-pyridyl)acrylic acid                     | 7340-22-9   | Activate Scientific GmbH    |
| No. | Name                          | CAS Number  | Source                          |
|-----|-------------------------------|-------------|---------------------------------|
| 50  | 3-(3-pyridyl)acrylic acid     | 19337-97-4  | Fisher Scientific GmbH          |
| 51  | 3-(4-pyridyl)acrylic acid     | 84228-93-3  | Fisher Scientific GmbH          |
| 52  | benzoic acid                  | 65-85-0     | Carl Roth GmbH & Co. KG         |
| 53  | o-toluic acid                 | 118-90-1    | Th. Geyer GmbH & Co. KG         |
| 54  | m-toluic acid                 | 99-04-7     | Th. Geyer GmbH & Co. KG         |
| 55  | p-toluic acid                 | 99-94-5     | Th. Geyer GmbH & Co. KG         |
| 56  | salicylic acid                | 20620-59-1  | Th. Geyer GmbH & Co. KG         |
| 57  | 3-hydroxybenzoic acid         | 99-06-9     | Th. Geyer GmbH & Co. KG         |
| 58  | 4-hydroxybenzoic acid         | 99-96-7     | Th. Geyer GmbH & Co. KG         |
| 59  | 2-methoxybenzoic acid         | 579-75-9    | Th. Geyer GmbH & Co. KG         |
| 60  | 3-methoxybenzoic acid         | 586-38-9    | Th. Geyer GmbH & Co. KG         |
| 61  | 4-methoxybenzoic acid         | 100-09-4    | Th. Geyer GmbH & Co. KG         |
| 62  | anthranilic acid              | 118-92-3    | Th. Geyer GmbH & Co. KG         |
| 63  | 3-aminobenzoic acid           | 99-05-8     | Th. Geyer GmbH & Co. KG         |
| 64  | 4-aminobenzoic acid           | 150-13-0    | Sigma-Aldrich                   |
| 65  | 2-nitrobenzoic acid           | 552-16-9    | Th. Geyer GmbH & Co. KG         |
| 66  | 3-nitrobenzoic acid           | 121-92-6    | Th. Geyer GmbH & Co. KG         |
| 67  | 4-nitrobenzoic acid           | 62-23-7     | TCI Deutschland GmbH            |
| 68  | 2-fluorobenzoic acid          | 445-29-4    | Th. Geyer GmbH & Co. KG         |
| 69  | 3-fluorobenzoic acid          | 455-38-9    | Th. Geyer GmbH & Co. KG         |
| 70  | 4-fluorobenzoic acid          | 456-22-4    | Th. Geyer GmbH & Co. KG         |
| 71  | 2-chlorobenzoic acid          | 118-91-2    | Th. Geyer GmbH & Co. KG         |
| 72  | 3-chlorobenzoic acid          | 535-80-8    | Th. Geyer GmbH & Co. KG         |
| 73  | 4-chlorobenzoic acid          | 74-11-3     | Th. Geyer GmbH & Co. KG         |
| 74  | 2,3-dihydroxybenzoic acid     | 303-38-8    | Th. Geyer GmbH & Co. KG         |
| 75  | 6-methylsalicylic acid        | 3147-64-6   | Fisher Scientific GmbH          |
| 76  | orsellinic acid               | 480-64-8    | Santa Cruz Biotechnology, Inc.  |
| 77  | piperonylic acid              | 94-53-1     | Th. Geyer GmbH & Co. KG         |
| 78  | 3-furoic acid                 | 488-93-7    | Th. Geyer GmbH & Co. KG         |
| 79  | propionic acid                | 79-09-4     | Carl Roth GmbH & Co. KG         |
| 80  | caproic acid/hexanoic acid    | 142-62-1    | Carl Roth GmbH & Co. KG         |
| 81  | octanoic acid                 | 124-07-2    | TCI Deutschland GmbH            |
| 82  | nonanoic acid                 | 112-05-0    | Th. Geyer GmbH & Co. KG         |
| 83  | undecanoic acid               | 112-37-8    | Th. Geyer GmbH & Co. KG         |
| 84  | lauric acid                   | 143-07-7    | Acros Organics BVBA             |
| 85  | tridecanoic acid              | 638-53-9    | TCI Deutschland GmbH            |
| 86  | myristic acid                 | 544-63-8    | Carl Roth GmbH & Co. KG         |
| 87  | palmitic acid                 | 57-10-3     | Carl Roth GmbH & Co. KG         |
| 88  | stearic acid                  | 57-11-4     | Sigma-Aldrich                   |
| TAL | 4-hydroxy-6-methyl-2-pyrone   | 675-10-5    | Sigma-Aldrich                   |
| 23a | hispidin                      | 555-55-5    | WVR International GmbH          |
| DK  | 5,6-dehydrokawain             | 15345-89-8  | Sigma-Aldrich                   |
| DDK | dihydro-5,6-dehydrokawain     | 3155-51-9   | Purified in this study          |

Table S1 (continued)

| No. | Name                          | CAS Number  | Source                          |
|-----|-------------------------------|-------------|---------------------------------|

**Note:** Purified in this study
| No. | Oligonucleotide | Sequence (5' - 3') |
|-----|----------------|------------------|
| 1   | pET28a_4CL_fw | ACGACCGAAAACCTGTATTTTCAGGGATCCGAGAAAGATACAAAACAG |
| 2   | pET28a_4CL_rv | GTGGTGTCGAGTGGGCCGCAAGCTTTTATTAATTTGGAAGCCACCCAG |
| 3   | pET28a_BZL_fw | TTTTTGAGTCAATGCGGAGGTCAGGTCAGCC |
| 4   | pET28a_BZL_rv | TTTTTAGCTTTTATACACAGCCAGCCTTACAC |
| 5   | pET28a_FadD_fw | TTTTTGGATCCCATCCCACAGGCCCGCATCTC |
| 6   | pET28a_FadD_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 7   | pET28a_FadK_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 8   | pET28a_FadK_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 9   | pET28a_FadA_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 10  | pET28a_FadA_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 11  | pET28a_AtoB_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 12  | pET28a_AtoB_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 13  | pET28a_YqeF_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 14  | pET28a_YqeF_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 15  | pJET_MatA_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 16  | pJET_MatA_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 17  | pJET_MatB_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 18  | pJET_MatB_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 19  | pMGE_4CL_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 20  | pMGE_BZL_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 21  | pMGE_FadD_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 22  | pMGE_FadD_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 23  | pMGE_FadK_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 24  | pMGE_FadK_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 25  | pMGE_FadA_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 26  | pMGE_FadA_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 27  | pMGE_FadI_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 28  | pMGE_FadI_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 29  | pMGE_KOMT1_fw | TTTTTGATCCATCCCACAGGCCCGCATCTC |
| 30  | pMGE_KOMT1_rv | TTTTTGATCCATCCCACAGGCCCGCATCTC |

Table S2. Oligonucleotides used in this study
### Table S3. Plasmids used in this study

| Plasmid                 | Relevant characteristics                                                                 | Source                           |
|-------------------------|------------------------------------------------------------------------------------------|----------------------------------|
| pET28a_H6TEV            | T7 promoter, His-tag, TEV, *kan*                                                        | [2]                              |
| pET28a_4CL              | pET28a_H6TEV, *4CL* Nicotiana tabacum                                                   | This study                       |
| pET28a_FadD             | pET28a_H6TEV, *fadD* *E. coli*                                                           | This study                       |
| pET28a_FadK             | pET28a_H6TEV, *fadK* *E. coli*                                                           | This study                       |
| pET28a_FadA             | pET28a_H6TEV, *fadA* *E. coli*                                                           | This study                       |
| pET28a_FadI             | pET28a_H6TEV, *fadI* *E. coli*                                                           | This study                       |
| pET28a_AtoB             | pET28a_H6TEV, *atoB* *E. coli*                                                           | This study                       |
| pET28a_YqeF             | pET28a_H6TEV, *yqeF* *E. coli*                                                           | This study                       |
| pJET 1.2                | T7 promoter, eco47IR, *amp*                                                             | Fisher Scientific                |
| pJET_MatA               | pJET 1.2, *matA* Rhizobium leguminosarum                                                  | This study                       |
| pJET_MatB               | pJET 1.2, *matB* Rhizobium leguminosarum                                                  | This study                       |
| pMGE-T7                 | pJET1.2, *T7* UC-ORI, *kan*                                                             | [3]                              |
| pMGE_4CL                | pMGE-T7, *4CL* Nicotiana tabacum                                                        | This study                       |
| pMGE_BZL                | pMGE-T7, *badA* Rhodopseudomonas palustris                                                | This study                       |
| pMGE_FadD               | pMGE-T7, *fadD* *E. coli*                                                                | This study                       |
| pMGE_FadK               | pMGE-T7, *fadK* *E. coli*                                                                | This study                       |
| pMGE_FadA               | pMGE-T7, *fadA* *E. coli*                                                                | This study                       |
| pMGE_FadI               | pMGE-T7, *fadI* *E. coli*                                                                | This study                       |
| pMGE_FadIK              | pMGE-T7, *fadK*, *fadI*                                                                 | This study                       |
| pMGE_FadDA              | pMGE-T7, *fadD*, *fadA*                                                                  | This study                       |
| pMGE_FadDI              | pMGE-T7, *fadD*, *fadI*                                                                  | This study                       |
| pMGE_FadKA              | pMGE-T7, *fadK*, *fadA*                                                                  | This study                       |
| pMGE_FadK1              | pMGE-T7, *fadK*, *fadI*                                                                  | This study                       |
| pMGE_FadD_C             | pMGE-T7, *fadD*, *4CL*                                                                  | This study                       |
| pMGE_FadD_4CL           | pMGE-T7, *fadD*, *4CL*                                                                  | This study                       |
| pMGE_FadI_BZL           | pMGE-T7, *fadI*, *badA*                                                                  | This study                       |
| pMGE_FadDA_KOMT1        | pMGE-T7, *fadD*, *fadA*, *KOMT1*                                                        | This study                       |
| pMGE_FadDI_KOMT1        | pMGE-T7, *fadD*, *fadI*, *KOMT1*                                                        | This study                       |
### Table S4. Strains used in this study

| Strain                  | Description                                             | Source                                      |
|-------------------------|---------------------------------------------------------|---------------------------------------------|
| *E. coli* BL21 (DE3)   | Host strain for enzymes expression                      | Lab collection                              |
| *E. coli* K-12 BW25113 | *lacI*, *rrnB*<sub>T14</sub>, *ΔlacZ*<sub>WJ16</sub>, *hsdR*<sub>514</sub>, *ΔaraBAD*<sub>Ang3</sub>, *ΔrhaBAD*<sub>LD78</sub> | Lab collection                              |
| ΔfadA                   | BW25113, ΔfadA, *kanR*                                  | Horizon Discovery Biosciences LTD           |
| ΔfadI                   | BW25113, ΔfadI, *kanR*                                  | Horizon Discovery Biosciences LTD           |
| ΔatoB                   | BW25113, ΔatoB, *kanR*                                  | Horizon Discovery Biosciences LTD           |
| ΔyqeF                   | BW25113, ΔyqeF, *kanR*                                  | Horizon Discovery Biosciences LTD           |
| ΔfadD                   | BW25113, ΔfadD, *kanR*                                  | Horizon Discovery Biosciences LTD           |
| ΔfadK                   | BW25113, ΔfadK, *kanR*                                  | Horizon Discovery Biosciences LTD           |
| ΔfadR                   | BW25113, ΔfadR, *kanR*                                  | Horizon Discovery Biosciences LTD           |
| *Pseudomonas fluorescens* | Wild-type strain, STI10619                           | Jena Microbial Resource Collection         |
| *Staphylococcus aureus* | Wild-type strain, ST033790                             | Jena Microbial Resource Collection         |
| *Enterococcus faecium*  | Wild-type strain, STI11237                             | Jena Microbial Resource Collection         |
| *Streptomyces hawaiiensis* | Wild-type strain, STI43082                           | Jena Microbial Resource Collection         |
| *Streptomyces griseus*  | Wild-type strain, STI40235                             | Jena Microbial Resource Collection         |
| *Streptomyces rapamycinicus* | Wild-type strain, ATCC29253                        | [9]                                         |
Table S5. Accession numbers of proteins used in this study

| Protein | Organism source                  | Genbank Accession No. |
|---------|----------------------------------|-----------------------|
| 4CL     | *Nicotiana tabacum*              | AAB18638.1            |
| BZL     | *Rhodopseudomonas palustris*     | CAE26105.1            |
| FadD    | *E. coli*                        | BAA15609.1            |
| FadK    | *E. coli*                        | BAA15470.2            |
| FadA    | *E. coli*                        | AAA67642.1            |
| FadI    | *E. coli*                        | BAA16197.2            |
| AtoB    | *E. coli*                        | BAA16020.1            |
| YqeF    | *E. coli*                        | BAE76913.1            |
| MatA    | *Rhizobium leguminosarum*        | AXA42395.1            |
| MatB    | *Rhizobium leguminosarum*        | AXA42396.1            |
| KOMT1   | *Piper methysticum*              | QCX36374.1            |
3. Supplementary Figures

Figure S1. Production of styrylpyrones was observed in *E. coli* expressing 4CL treated with phenylpropionic acids. LC-HRMS EIC showing the production of styrylpyrones when A) cinnamic acid and B) 3-phenylpropionic acid were individually fed to BL21 and BL21 expressing 4CL. 4CL, 4-coumaroyl-CoA ligase from *Nicotiana tabacum*. 
**Figure S2. Purified enzymes used in this study.** Coomassie-stained SDS-PAGE of 2 µg of the purified Protein. The 4-coumaroyl-CoA ligase (4CL) was from *Nicotiana tabacum*; Benzoate-CoA ligase (BZL) was from *Rhodopseudomonas palustris*; Long-chain-fatty-acid-CoA ligase (FadD), medium-chain-fatty-acid-CoA ligase (FadK) and thiolases FadA, FadI, AtoB, YqeF were from *E. coli*; Malonyl-CoA decarboxylase (MatA) and malonyl-CoA synthetase (MatB) were from *Rhizobium leguminosarum*. The expected molecular weights of enzymes with N terminal 6 x His-tag calculated by tool from ExPASy (http://web.expasy.org/compute_pi/): 4CL (theoretical Mw: 62.5 kDa), BZL (theoretical Mw: 59.7 kDa), FadD (theoretical Mw: 65.3 kDa), FadK (theoretical Mw: 65.7 kDa), FadA (theoretical Mw: 43.9 kDa), FadI (theoretical Mw: 49.5 kDa), AtoB (theoretical Mw: 43.3 kDa), YqeF (theoretical Mw: 44.0 kDa), MatA (theoretical Mw: 54.3 kDa), MatB (theoretical Mw: 57.6 kDa).
Figure S3. LC-HRMS detection of tetraacetic acid lactone and diketide intermediates. The EICs of other products (tetraacetic acid lactone and diketide intermediates) produced by the in vitro enzymatic reactions shown in Figure 2 A. As previously reported, the CoA-ester intermediates showed broad peaks.^[10]
Figure S4. Summary of in vitro reactions catalysed by *E. coli* thiolase enzymes. Thiolas FadA, FadI, AtoB and YqeF use up to two units of acetyl-CoA for non-decarboxylative Claisen condensations. A) When acetoacetyl-CoA was used as starting substrate, FadA, FadI, AtoB and YqeF catalysed both one and two rounds of condensations. However, YqeF produced TAL and tetraacetilic acid lactone only in very low yields. B) When using acetyl-CoA as starting substrate, FadA and FadI catalysed two rounds of condensation and synthesized TAL; AtoB used both one and two units of acetyl-CoA to form acetyl-CoA and TAL, respectively; while YqeF only used one unit of acetyl-CoA to form acetoacetyl-CoA. 4CL esterified C) cinnamic acid and D) 3-phenylpropionic acid to corresponding CoA esters, which were then used only by FadA and FadI to form α-pyrones 1a and 32a. E) Benzoic acid was esterified by BZL to benzoyl-CoA. Then only FadA used two units of acetyl-CoA to form α-pyrones 52a. AtoB and YqeF used one unit of acetyl-CoA to form diketide intermediate. F) FadD was used to esterify hexanoic acid. FadA and FadI catalysed two rounds of
condensations to form α-pyrones 80a, while AtoB and YqeF only catalysed one round of condensation to form diketide intermediate.
Figure S5. The production of 6-styryl- and 6-dihydrostyryl-α-pyrone in vitro with the purified ligase 4CL and thiolase FadA/FadI. Starting substrates 4, 17, 23 and 29 contain both cis and trans isomers, while 16 and 24 were only trans isomers, but yangonin-like compounds have been reported to spontaneously isomerize between cis and trans configurations in solution in the presence of light\cite{11}. LC-HRMS analysis of the hispidin standard also showed double peaks as in 23a. This explains the detection of two different chromatographic peaks with the same mass in several reactions.
Figure S6. The production of 6-aryl-α-pyrone s \textit{in vitro} with the purified ligase BZL and thiolase FadA/FadI.
Figure S7. The production of 6-alkyl-α-pyrones in vitro with the purified ligase FadD and thiolase FadA/FadI.
Figure S8. The production of 6-alkyl-α-pyrone s in vitro with the purified ligase FadK and thiolase FadA/FadI.
Figure S9. The production of bioactive styrlypyrones in vivo. LC-HRMS EIC showing the production of 6-styryl-α-pyrone (16a, 17a, 22a and 29a) and 6-dihydrostyrly-α-pyrone (37a, 38a, 41a and 43a) from E. coli strains containing FadD and FadA (or Fadl) expressed from pMGE-T7 vector fed individually with 16, 17, 22, 29, 37, 38, 41 and 43. The reason for the detection of two different chromatographic peaks with the same mass was explained in Figure S5.
Figure S10. α-pyrones were observed in different bacterial species without precursor feeding. We observed that most of tested bacteria produced TAL without feeding any precursor. In the strain *E. faecium*, although no TAL production was detected, we observed the production of fatty acid-derived pyrones (80a and 81a). The *in vitro* enzymatic reactions using the purified FadA and FadD were used as positive controls.
Figure S11. Calibration curves for the quantification of kavalactones DK and DDK. Calibration curves for DK (A) and DDK (B) determined using peak areas of HRLC-HRMS measurements of samples with concentration ranges from 0 to 2.5 μg/mL and 0 to 31.25 μg/mL, respectively. All samples were measured in triplicates and error bars represent standard deviations.
Figure S12. $^1$H NMR (600 MHz, methanol-$d_4$) spectrum of DDK
Figure S13. $^{13}$C and DEPT135 NMR (150 MHz, methanol-$d_4$) spectrums of DDK
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