Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Isocoumarin formation by heterologous gene expression and modification by host enzymes

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Experimental procedures

1.1 General Experimental Procedures

All chemicals were purchased from Sigma-Aldrich. High-resolution mass spectrometric data were generated by using a microTOF-Q III mass spectrometer with an ESI source (Bruker Daltonics, Bremen, Germany) connected to an Agilent HPLC series 1260 (Böblingen, Germany), which is equipped with a photodiode array detector. Separation was carried out on an Eclipse XDB-C18 column (4.6 × 150 mm, 5 *µ*m, Agilent, Germany). NMR spectra were recorded on a JEOL ECA-500 MHz spectrometer (JEOL, Tokyo, Japan). The spectra were processed by using the software MestReNova 6.1.0 (Metrelab). NMR data are given in Table S1 and spectra in Figures S3 – S5.

1.2 Strains, Media, and Culture Conditions

Escherichia coli DH5*α* (Invitrogen) was used for cloning and plasmid propagation and grown in lysogeny broth (LB) at 37 °C. For cultivation on solid medium, 1.5% (w/v) agar was added. 50 *µ*g/mL carbenicillin were used for selection of the recombinant *E. coli* strains.

P. crustosum strain PRB-2 was cultivated under static conditions at 25 °C in potato dextrose broth (PDB, Sigma-Aldrich). *A. nidulans* LO8030 was cultivated at 37 °C in liquid minimal medium (LMM) supplemented with riboflavin, pyridoxine, uridine and uracil as reported previously.[1] For cultivation on solid medium, 1.5% (w/v) agar was added to the respective medium. Cultivation of *A. nidulans* strain PX001 (*gpdA::pcr9304::AfpyrG* in *A. nidulans* LO8030) and negative control (*gpdA::AfpyrG* in *A. nidulans* LO8030) were done on rice supplemented with riboflavin and pyridoxine at 25 °C.

1.3 Genetic Manipulations

For isolation of genomic DNA, *P. crustosum* and *A. nidulans* were cultivated in PDB or LMM for 2 days at 25 °C. Genomic DNA was isolated according to the method described previously.[2] Phusion high-fidelity DNA polymerase (New England Biolabs) was used for PCR amplifications on a T100 thermal cycler from Bio-Rad. Construction of the heterologous expression vector was done by homologous recombination in *E. coli*.[3] The construct pPX001 for heterologous expression in *A. nidulans* with afpyrG as a selection marker was based on the plasmid vector pYH-*w*A-pyrG.[5] Briefly, the vector backbone was linearized by cut with *Nhe*I followed by SAP treatment with subsequent purification *via* a HiYield PCR Clean-up and GelExtraction Kit (SLG Südlaborbedarf). For creation of pPX001, *pcr9304* with the accession number MT451935 (GenBank) including its downstream region of 633 bp was PCR amplified from genomic DNA of *P. crustosum* PRB-2 with the primer pairs An-9304-For/Rev (An-9304-For: TATATTCATCTTCCCATCCAAGAACCTTTAATCATGCGTCGGCCAAATC; An-9304-Rev: CATATTTCGTCAGACACAGAATAACTCTCGGTGACAATTCTCACCCTTG). The underlined sequences in the primers are homologous regions with the vector for recombination. Subsequently, the PCR fragment and linearized vector were mixed and transformed into *E. coli* DH5*α*. The assembled plasmid pPX001 was confirmed by enzyme restriction. 4 µg of this construct were linearized with *Swa*I and transformed into *A. nidulans* LO8030 according to the protocol by Yin *et al*.[5] 65 Potential transformants were obtained after uracil and uridine autotrophy selection. The primers 9304-F (CCTTTTCGAGTAACAGTCGC) and 9304-R (ATGCTTGATTACTGCCTTTTG) target a 1718 bp partial fragment in *pcr9304* and

were used for transformant verification. All of the 10 selected transformants were confirmed by PCR amplification.

1.4 Product Analysis, Large-Scale Fermentation, Extraction and Metabolite Isolation

A. nidulans strains were cultivated on rice medium at 25°C for 14 days for LC-MS analysis of the secondary metabolite production. For a small-scale analysis, 3 mL of the culture was extracted with equal volume of ethyl acetate. The organic phase was dried in vacuo using a Speedvac and the residue was dissolved in a mixture of MeOH and H_2O (19 : 1) for LC-MS analysis.

To isolate the accumulated products, *A. nidulans* PX001 was cultivated in seven 1 L flasks containing 100 g rice each and 150 mL H_2O with appropriate nutrition as supplement at 25°C for 21 days. After extraction with 3 L ethyl acetate for three times and concentration of the organic phases under reduced pressure, 3.4 g crude extract were obtained. Silica gel column chromatography of the crude extract by using dichloromethane / MeOH (100 : 1, 50 : 1, 10 : 1 and 0 : 1, *v/v*) as elution solvents, yielded 48 fractions. Further purification of fraction 8 on a semipreparative HPLC (Agilent series 1200 HPLC, Böblingen, Germany), with an Agilent Eclipse XDB-C18 column (9.4 × 250 mm, 5 *μ*m) and CH_3CN / H_2O (55 : 45, flow rate of 2.5 mL/min) as solvents, resulted in compound **1** (9.0 mg) in high purity. Purification of the combined fractions 25 – 30 on the same HPLC equipment by using CH_3CN / H_2O (35 : 65, flow rate of 2.5 mL/min) as solvents led to 7.0 mg of **2** and 6.5 mg of **3**.

*6,8-Dihydroxy-3-methylisocoumarin (**1**)*: yellow powder; HRMS (m/z): (ESI/[M + H]$^+$) calcd. for $C_{10}H_9O_4$, 193.0495, found 193.0493.

*6,8-Dihydroxy-3-hydroxymethylisocoumarin (**2**)*: white needle powder; HRMS (m/z): (ESI/[M + H]$^+$) calcd. for $C_{10}H_9O_5$, 209.0444, found 209.0451.

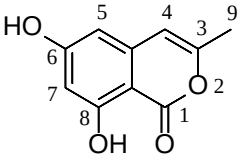
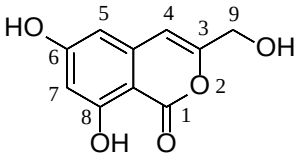
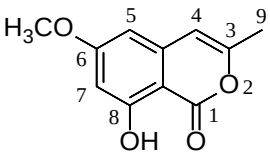
*6-Methoxy-8-hydroxy-3-methylisocoumarin (**3**)*: yellow oil; HRMS (m/z): (ESI/[M + H]$^+$) calcd. for $C_{11}H_{11}O_4$, 207.0652, found 207.0663.

1.5 Precursor Feeding in *A. nidulans* LO8030

A. nidulans LO8030 was cultured on solid rice medium (2 g rice, 3 mL H_2O, containing the appropriate supplements) at room temperature at a static condition. After three days, 10 *µ*L of 6,8-dihydroxy-3-methylisocoumarin (**1**) as a 1 M stock solution in DMSO was added to the rice culture, resulting in a final concentration of 0.4 mM. After cultivation for 7 further days, the secondary metabolites were extracted with ethyl acetate, dissolved in a mixture of MeOH and H_2O (19 : 1) and analyzed by LC-MS.

Supplementary Tables

Table S1. ^{1}H NMR data of compounds **1, 2** and **3** (DMSO-d_6)

Compound	 6,8-Dihydroxy-3-methylisocoumarin (**1**)[4]	 6,8-Dihydroxy-3-hydroxymethylisocoumarin (**2**)[4]	 6-Methoxy-8-hydroxy-3-methylisocoumarin (**3**)[4]
Position	δ_H, multi., *J* in Hz	δ_H, multi., *J* in Hz	δ_H, multi., *J* in Hz
4	6.47, q, 1.0	6.61, s	6.27, q, 1.2
5	6.33, d, 2.5	6.43, d, 2.1	6.43, d, 2.2
7	6.30, d, 2.5	6.34, d, 2.1	6.33, d, 2.2
9	2.22, d, 1.0	4.25, d, 1.0	2.13, d, 1.2
6-OH	10.79, brs	-	-
8-OH	10.95, s	10.93, brs	10.66, brs
9-OH	-	5.57, brs	-
6-OCH_3	-	-	3.81, s

The NMR data of the isolated compounds correspond very well to those reported in the literature.[4]

Supplementary Figures

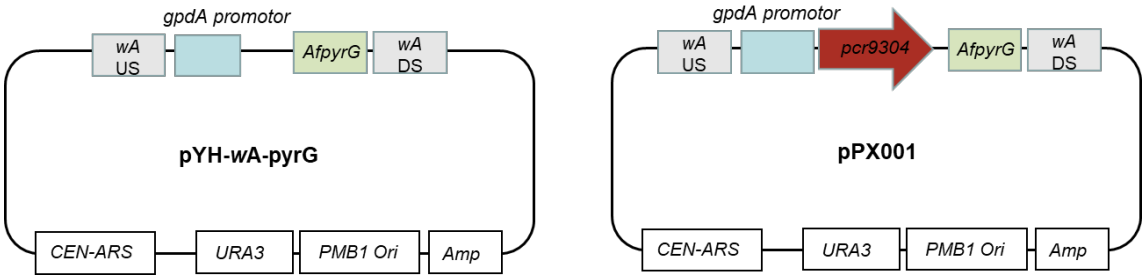


Figure S1. Constructs used for heterologous expression in *A. nidulans*

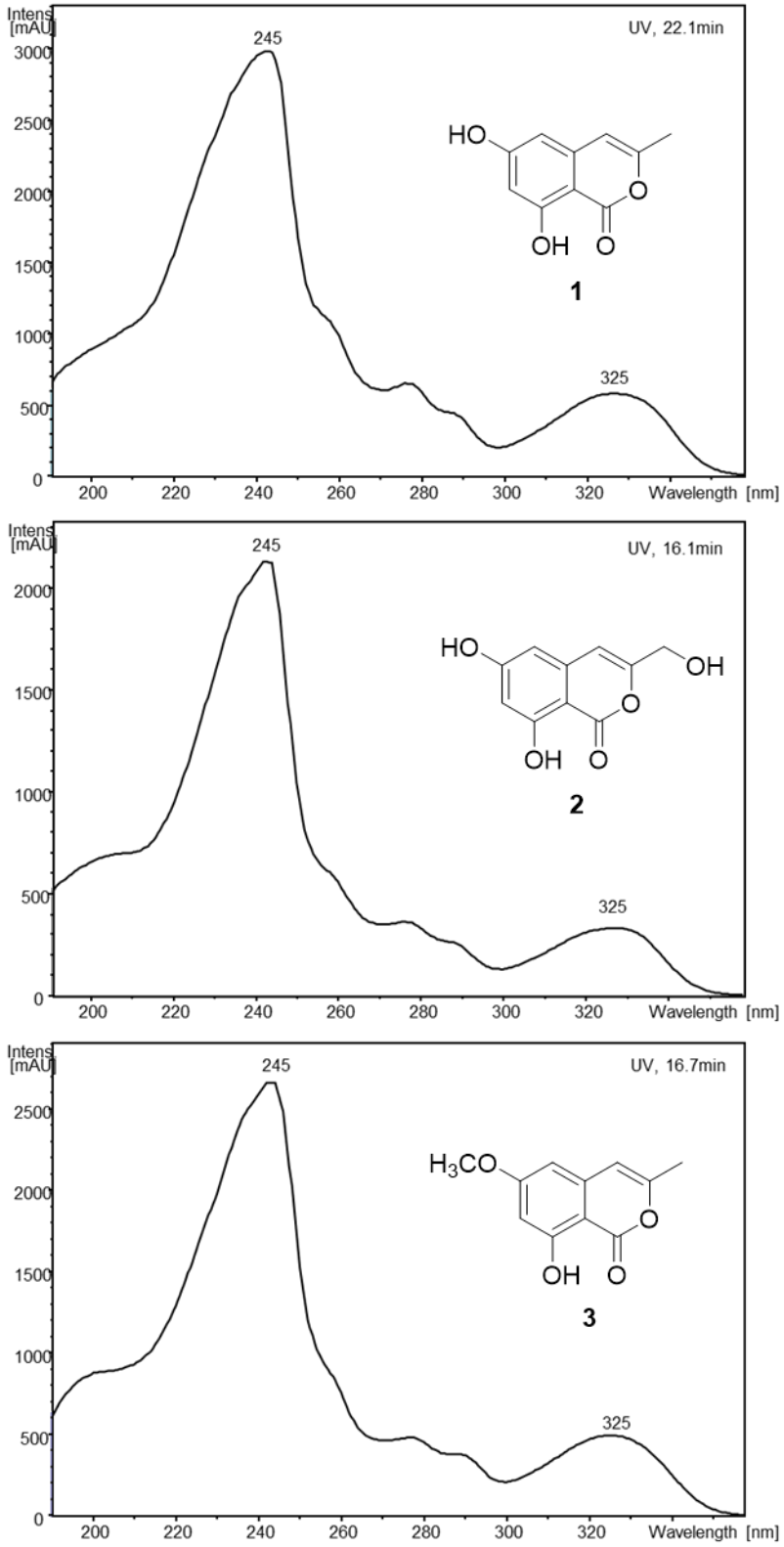


Figure S2. UV spectra of compounds **1**, **2** and **3**

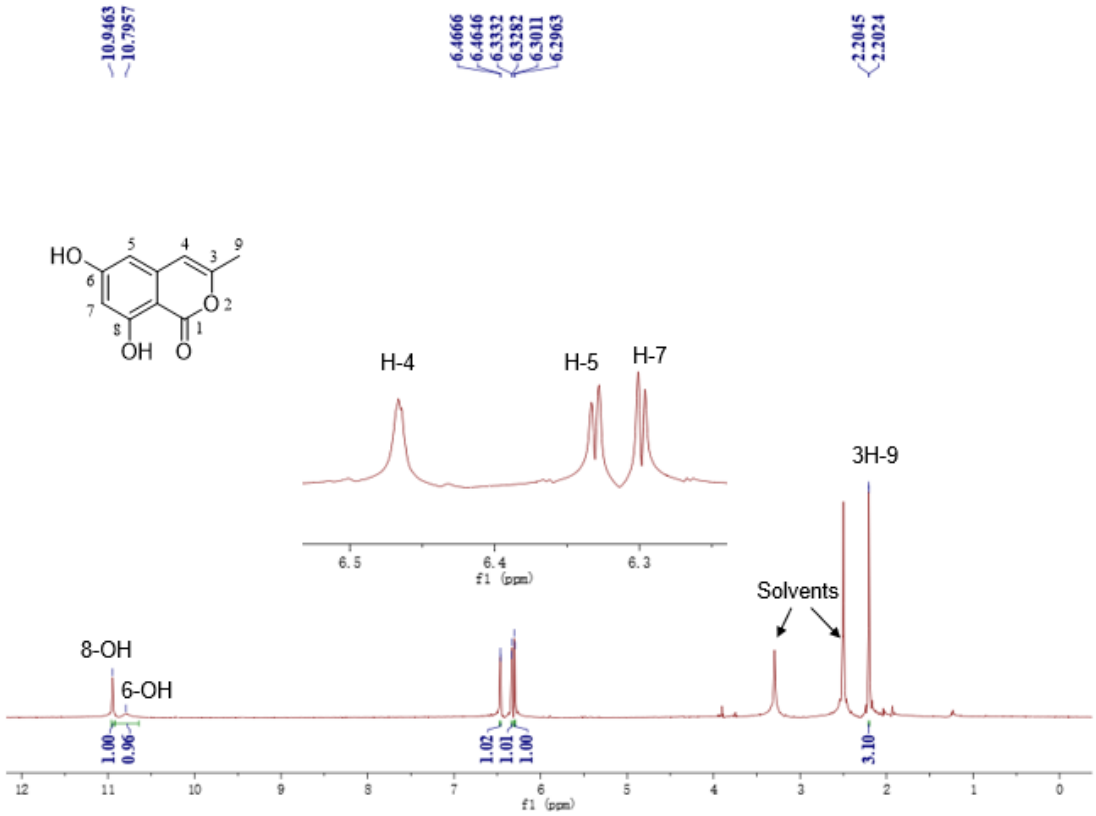


Figure S3. ^{1}H NMR spectrum of compound **1** in DMSO-d_6 (500MHz)

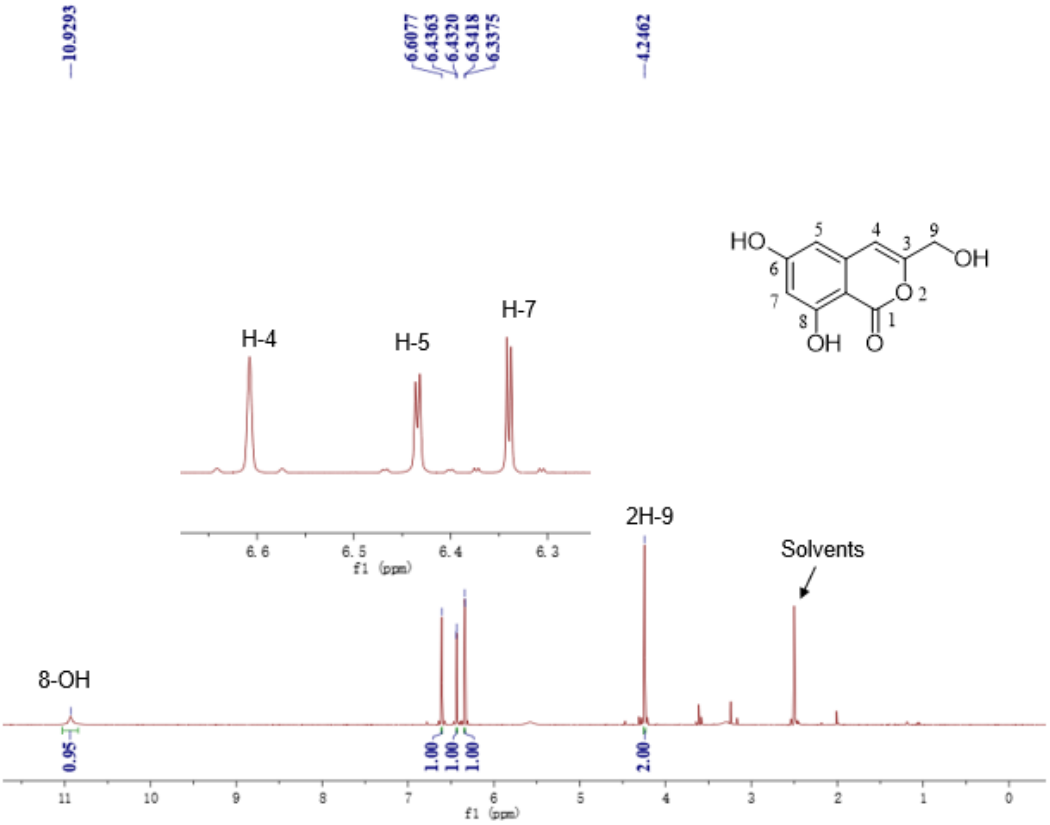


Figure S4. ^{1}H NMR spectrum of compound **2** in DMSO-d_6 (500MHz)

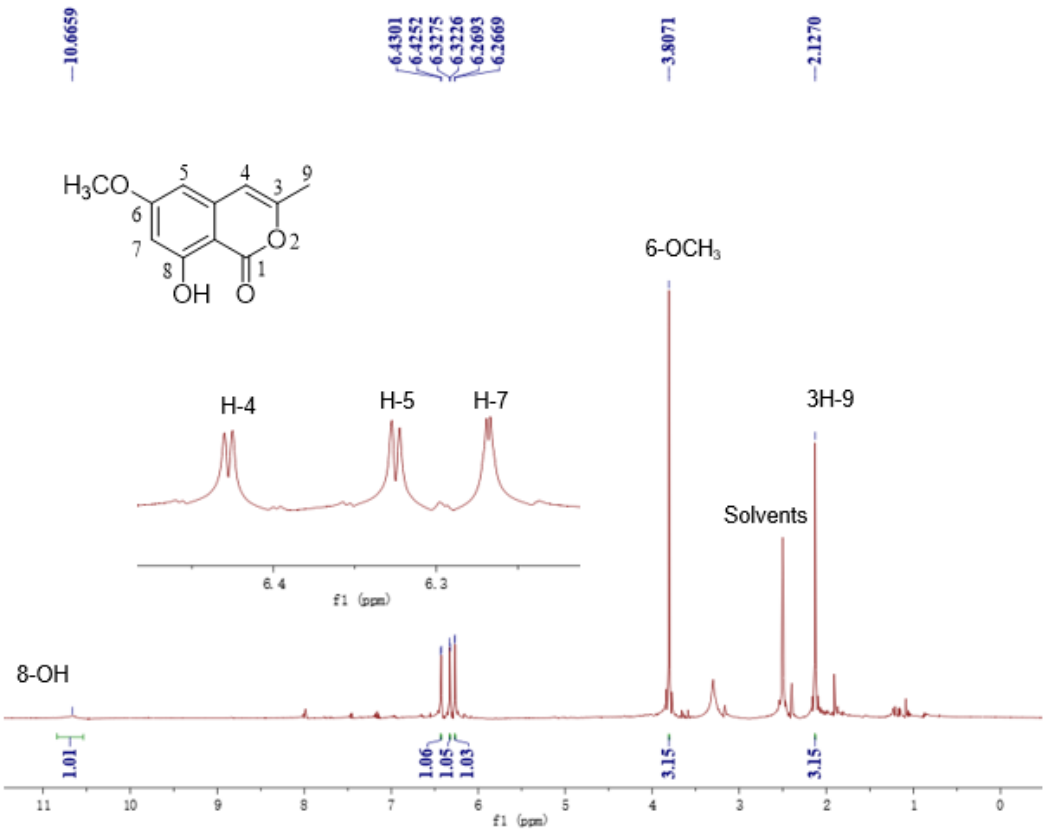


Figure S5. ^{1}H NMR spectrum of compound **3** in DMSO-d_6 (500MHz)

Supplementary References

1 Y. M. Chiang, M. Ahuja, C. E. Oakley, R. Entwistle, A. Asokan, C. Zutz, C. C. Wang, and B. R. Oakley, *Angew. Chem. Int. Ed. Engl.*, 2016, **55**, 1662-1665.

2 J. Fan, G. Liao, F. Kindinger, L. Ludwig-Radtke, W.-B. Yin, and S.-M. Li, *J. Am. Chem. Soc.*, 2019, **141**, 4225-4229.

3 A. P. Jacobus and J. Gross, *PLoS. One.*, 2015, **10**, e0119221.

4 H. Kumagai, M. Amemiya, H. Naganawa, T. Sawa, M. Ishizuka, and T. Takeuchi, *J. Antibiot.*, 1994, **47**, 440-446.

5 W. B. Yin, Y. H. Chooi, A. R. Smith, R. A. Cacho, Y. Hu, T. C. White, and Y. Tang, *ACS Synth. Biol.*, 2013, **2**, 629-634.