Investigating the biosynthesis of Sch-642305 in the fungus *Phomopsis* sp. CMU-LMA

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4. Supplementary references
1. General experimental procedures

All reagents and chemicals were purchased from Sigma Aldrich, Carl Roth or Thermo Fischer Scientific. All solvents used for HPLC were analytical grade. General molecular biology procedures were performed according to standard protocols or following the manufacturer’s instructions in case of commercially obtained equipment/kits. For polymerase chain reaction either OneTag polymerase (NEB; analytical purposes) or Q5 polymerase (NEB; cloning purposes) was used. All restriction enzymes deployed in this study were obtained from NEB and used according to the manufacturer’s protocol. All media used was prepared in deionized water and sterilized at 126 °C for at least 20 min. NMR analysis, HRMS analysis and analytical as well as preparative LC/MS was performed as published previously. All concentrations are w/v unless otherwise specified. (1)

1.1. Bioinformatics

Pairwise sequence identities of proteins were determined using Clustal Omega in default settings. (2) The putative *psch* BGC was identified in the publicly available genome of *P. verrucosum* (NCBI: BioProject PRJNA276626). ORFs within the cluster were manually annotated using BLAST(3) and Softberry FGENESH 2.6(4) with default settings and using *Penicillium chrysogenum* as reference organism. Domain analysis of *schPKS* was performed using the InterPro(5) tool.

1.2. Media and solutions

- Czapek Dox with sorbitol (CD+S; Czapek Dox broth 3.5%, D-sorbitol 18.22%, agar 1.5%)
- Czapek Dox with sorbitol, soft agar (CD+S soft agar; Czapek Dox broth 3.5%; D-sorbitol 18.22%; agar 0.8%)
- CD/S agar (3.5% Czapek Dox broth; 1 M sorbitol; 0.05% adenine; 0.15% methionine; 0.1% (NH$_4$)$_2$SO$_4$)
- CZD/S agar (Czapek Dox broth 3.5%; D-sorbitol 18.22%; ammonium sulfate 0.10%; adenine 0.05%; L-methionine 0.15%; agar 1.5%)
- CZD/S1 agar (-Met) (Czapek Dox broth 3.5%; D-sorbitol 18.22%; ammonium sulfate (0.10%; L-methionine (optional) 0.15%; agar 1.5%)
- CZD/S softagar (Czapek Dox broth 3.5%; D-sorbitol 18.22%; ammonium sulfate 0.10%; adenine 0.05%; L-methionine 0.15%; agar 0.8%)
- CZD/S1 (-Met) Softagar (Czapek Dox broth 3.5%; D-sorbitol 18.22%; ammonium sulfate 0.10%; L-methionine (optional) 0.15%; agar 0.8%)
- CD1 agar (Czapek Dox broth 3.5%; NaCl 4.68%; ammonium sulfate 0.10%; adenine 0.05%; L-methionine 0.15%; agar 1.5%)
- CD2 agar (-Met) (Czapek Dox broth 3.5%; NaCl 4.68%, ammonium sulfate 0.10%; L-methionine 0.15%; agar 1.5%)
- DPY (dextrin from potato starch 2%; polypeptone 1%; yeast extract 0.5%; KH$_2$PO$_4$ 0.5%; MgSO$_4$ 0.05%; agar 2.5%)
- LB agar (yeast extract 0.5%; tryptone 1%; NaCl 0.5%; agar 1.5%)
- Malt extract (ME; malt extract 1.28%; peptone ex soya 0.08%; glycerol 0.24%; detrin from potato starch 0.28%; agar 1.5%)
- M10 medium (Ca(NO$_3$)$_2$ 1.2 mM; KNO$_3$ 0.79 mM; KCl 0.87 mM; MgSO$_4$ 3 mM; Na$_2$HPO$_4$ 0.14 mM; sucrose 87.6 mM; ammonium tartrate 27.1 mM; FeCl$_3$ 7.4 µM; MnSO$_4$ 30 µM; ZnSO$_4$ 8.7 µM; H$_3$BO$_3$ 22 µM; KI 4.5 µM; pH 5.5)
- PDB medium (potato infusion 200 g L$^{-1}$; dextrose 20 g L$^{-1}$; pH 5.1)
- PDB agar (potato infusion 200 g L$^{-1}$; dextrose 20 g L$^{-1}$; pH 5.1; agar 1.5%)
- Supplement mixture minus uracil (SM-URA; yeast nitrogen base 0.17%; (NH$_4$)$_2$SO$_4$ 0.5%; D(+)-glucose monohydrate 2%; complete supplement mixture minus uracil 0.077%; agar 1.5%)
- YPAD (yeast extract 1%; tryptone 2%; D(+)-glucose monohydrate 2%; adenine 0.03%)
1.3. Strains and culture conditions

*Phomopsis* sp. CMU-LMA was provided by Dr. Jamal Ouazzani. Typically, the fungus was cultured on PDB agar at 28 °C. Formation of spores was observed after 10 d cultivation and spore suspensions containing 25% glycerol allowed long-term storage at -80 °C. For secondary metabolite production *Phomopsis* sp. CMU-LMA was inoculated into PDB medium (pH 5.2) and kept at 28 °C, 110 rpm for up to 8 d (in particular used for *Phomopsis* sp. CMU-LMA wildtype and all KO strains except *Phomopsis* sp. CMU-LMAΔschR3). Alternatively, *Phomopsis* sp. CMU-LMA was inoculated into M1D medium and kept at 28 °C and static conditions for 10 d (for *Phomopsis* sp. CMU-LMAΔschR3). *Phomopsis* KO strains were always cultivated alongside a WT strain as positive control. *Aspergillus oryzae* NSAR1 was typically cultured on DPY agar (28 °C). Spores were harvested after 7d and mixed with glycerol to a final concentration of 25%, allowing long-term storage at –80 °C. For secondary metabolite production spores were inoculated into 500 ml baffled flasks containing 100 ml DPY medium and kept at 28 °C and 110 rpm for 7 d. *E. coli* TOP-10 cells were grown on solid or liquid LB medium supplemented with the appropriate selection marker. Typically, cultures were incubated at 37 °C and 225 rpm for 16 h. Glycerol stocks containing 50% glycerol (v/v) were prepared for long-term storage at -80 °C. *Saccaromyces cerevisiae* CEN.PK2 was cultured on YPAD agar at 30 °C for 2-3 d. Alternatively, a single colony was inoculated into 10 ml liquid YPAD medium and grown at 30 °C and 200 rpm for 16 h.

1.4. Chemical analysis of cultures

Mycelia of *Phomopsis* sp. CMU-LMA WT and KO strains was blended in the supernatant of producing cultures. Cell debris was removed by filtration and the filtrate was extracted two times with ethyl acetate (filtrate : ethyl acetate 1:5). Organic layers were combined and dried over anhydrous MgSO₄. Solvent was removed under reduced pressure and the crude extract was dissolved in acetonitrile for LC/MS analysis.

For *Aspergillus oryzae* NSAR1 mycelium and supernatant was separated by Büchner filtration. The supernatant was acidified to pH 2 (2M HCl), mixed with equal amounts of ethyl acetate and extracted twice. In parallel, the mycelium was stirred in acetone (100 ml per 100 ml culture) for 1h (RT) prior to evaporation of the solvent under reduced pressure. 100 ml ddH₂O was added to the remaining mycelium fraction, acidified to pH 2 (2M HCl) and extracted twice with equal amounts of ethyl acetate. Combined ethyl acetate extracts from supernatant and cells were dried over anhydrous magnesium sulfate and organic solvent was removed in vacuo. Crude extracts were dissolved in methanol to a final concentration of 10 mg ml⁻¹.

1.5. Isotopic feeding experiments

Sodium [1-¹³C]-labelled acetate was obtained from Sigma Aldrich. Subsequently, growing cultures of *Phomopsis* sp. CMU-LMA were supplemented with the labelled isotope to a final concentration of 15 mM.

1.6. Yeast homologous recombination

Assembly of Knockout plasmids and expression plasmids was routinously achieved by yeast homologous recombination. The uracil auxotrophic *S. cerevisiae* CEN.PK2 strain was used. Typically, a single colony was inoculated into 10 ml YPAD medium and grown overnight at 30 °C and 160 rpm. This seed culture was mixed with 40 ml fresh YPAD medium and kept at 30 °C and 160 rpm for 4-5h. Next, the cells were collected by centrifugation and washed twice with ddH₂O prior to final resuspension in 1 ml ddH₂O. 100 µL were centrifuged (13,000 x g, 4 °C, 1 min) and resuspended in the transformation solution (240 µL PEG 3350 (50% w/v), 36 µL LiOAc (1M), 50 µL denatured salmon sperm DNA (2 mg ml⁻¹) and 34 µL DNA master mix). The DNA master mix contained linearized vector and DNA insert with a 5’ and 3’ overlap of 30 bp with the cut sites of the vector (fragments). Vectors were either linearized by *NotI* (pE-YA) or *Ascl* (pTYGSarg/ade/met). The resulting mixture of DNA and yeast was incubated at 42 °C for 50 min. After centrifugation (13,000 x g, 4 °C, 1 min) the pellet was resuspended in 1 ml ddH₂O
and plated on SM-URA agar. After incubation at 30 °C for 2-3 days plasmid was isolated using the Zymoprep Yeast Plasmid Miniprep kit, transformed into *E. coli* TOP10 or *E. coli* ccdb cells, re-isolated from bacterial cells and submitted to DNA sequencing. DNA sequencing was performed by *Eurofins Genomics*.

### 1.7 Construction of *Phomopsis* sp. CMU-LMA knockout plasmids

KO-cassettes for gene disruption in *Phomopsis* sp. CMU-LMA were designed as displayed in supplementary figure S5. KO-cassettes were composed of three DNA fragments previously amplified by polymerase chain reaction: the hygromycin resistance cassette (here named HygR; comprising P_gdpA + hph, ~ 4 KBp) and two gene specific sequences homologous to the target gene (Target Left T_L and Target Right T_R; each ~ 1 KBp). HygR was designed to have overlaps with T_L and T_R at the 3´ and 5´ end respectively. T_L and T_R were designed to have additional overlaps with the destination vector pE-YA. Overlaps comprised 30 nt. The KO-cassette was cloned into the shuttle vector pE-YA by yeast homologous recombination. Primers used for the generation of KO plasmid are listed in supplementary table S8. Successful generation of KO plasmids was confirmed by DNA sequencing.

### 1.8 Construction of *A. oryzae* expression vectors

Total RNA was extracted from *Phomopsis* sp. CMU-LMA using oligo (dT) nucleotides and the RevertAid Premium Transcriptase kit (Thermo Fischer) was used according to the manufacturer’s instructions to transcribe RNA into cDNA. For *schPKS* the Maxima Reverse Transcriptase from NEB was used. Intron free cDNA was used for PCR amplification of biosynthetic genes. *schPKS* was assembled in the pE-YA vector and shuttled into the pTYGS expression vector by gateway cloning (Invitrogen Gateway LR Clonase II enzyme mix; manufacturer’s protocol with halved reaction scale).

For assembly in pE-YA *schPKS* (about 8 kb) was amplified in four fragments, each about 2 kb. Yeast homologous recombination yielded plasmid KELIII02A(No2_3) and DNA sequencing identified the presence of four predicted introns, suggesting that the cDNA used for amplification was contaminated with traces of gDNA. To remove the introns *schPKS* was reassembled in pE-YA. For this, *schPKS* was amplified from KELIII02A(No2_3) in seven fragments, omitting the predicted intron sequences and yielding plasmid KELIII07. Sequencing revealed that introns were indeed absent but that a point mutation was present (insertion of alanine at position 2490) leading to an undesired frame shift. The point mutation was manually repaired by yeast homologous recombination using KpnI digested KELIII07 and an amplified patch from KELIII02A(No2_3) having the correct sequence (supplementary figure S40). Reassembled plasmid KELIII08 was confirmed by sequencing and shuttled into the pTYGS expression plasmid, yielding KELIII08a, using gateway cloning. Yeast homologous recombination was used to clone the tailoring biosynthetic genes into the respective multigene expression vectors pTYGSarg/ade/met under control of a constitutive promoter (either peno, padh, pgdpA; see supplementary figure S41). Expression vector KELIII08C was revealed to possess two point mutations in *schR3* causing a frame shift resulting in inactive *schR3*. This vector was only used in experiments lacking *schR3*. Details for construction of expression plasmids are outlined in supplementary table S10 and oligonucleotides are noted in supplementary table S11.

### 1.9 Transformation of *Phomopsis* sp. CMU-LMA

For transformation of *Phomopsis* sp. CMU-LMA a protoplast mediated protocol was established. Mycelia from growing plates was inoculated into 50 ml PDB medium and incubated at 28 °C in static conditions for up to 5d. Cells were collected by filtration through sterile miracloth. Protoplast formation was accomplished by digestion of young mycelia with a mixture of lysing enzymes extracted from *Trichoderma harzianum* (10 mg ml⁻¹, Sigma Aldrich) and driselase from *Basidiomycetes* spp. (5 mg ml⁻¹, Sigma Aldrich) in osmotic buffer (0.8 M NaCl). The protoplasting solution was kept at 30 °C for 2-3 hours while being slowly inverted. Protoplasts were released from hyphal strands by careful pipetting followed by filtration through sterile miracloth. After centrifugation (3000 x g, 5 min, 4 °C) the pelleted protoplasts were resuspended in 100 µL Solution 1 (0.8M NaCl; 10 mM CaCl₂; 50 mM Tris-HCl pH 7.5)
and the concentration was assessed microscopically using a hemocytometer. Typically, 10 µg DNA were added to the protoplast solution and after incubation on ice for 2 minutes 1 ml of Solution II (60% w/v PEG3350; 0.8M NaCl; 10 mM Tris-HCl pH 7.5) was added and incubated at RT for another 20 minutes. Finally, 5 ml of molten CZD/S agar (at 50 °C) was added and the mixture was spread evenly on a 10 cm Petri dish. Plates were incubated at 28 °C overnight for cell wall regeneration and then overlayed with 10 ml CD agar containing Hygromycin B at 100 µg ml$^{-1}$. Emerging single colonies were transferred to secondary plates (CD agar with Hygromycin B at 100 µg ml$^{-1}$). Colonies that grew healthily on secondary plates were transferred to medium without antibiotic and assessed for correct integration of the KO cassette.

1.10. Assessment of targeted gene disruption

Successful disruption of sch biosynthetic genes was confirmed using polymerase chain reaction. For this, gDNA was isolated from *Phomopsis* sp. CMU-LMA KO strains using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma Aldrich). Two PCR reactions were performed on each sample and WT gDNA as control. Whole PCR (wPCR) reveals successful disruption as it amplifies the entire target gene cassette (in case of schPKS a 1 kbp region within the internal region that is deleted upon successful gene disruption). In case of WT gDNA wPCR gives the correct insert size. In case of KO strains wPCR yields a larger PCR product as the whole disruption cassette is amplified, including the *hph* resistance gene. External PCR (extPCR) reveals whether recombination occurred at the desired site: primers are designed to amplify from 5’ end of the *hph* resistance gene to genomic DNA flanking the target gene. extPCR only gives a product in case of successful disruption in the correct locus. It does not give a PCR product using WT gDNA, as WT gDNA lacks the *hph* gene. For wPCR the gene specific primers Tlf and TR Re were used (compare oligonucleotides used for construction of KO plasmids). For external PCR validation gene specific primers (supplementary table S9) in combination with either primer HygRP7.2 Fw or HygRP6.2 Re (both binding to hygromycin gene cassette) were used.

1.11. Biotransformation of SCH 1 by *Aspergillus oryzae* NSAR1

*Aspergillus oryzae* NSAR1 from a sporulating DPY agar plated was inoculated into 500 ml baffled flasks containing 100 ml DPY medium and kept at 28 °C, 110 rpm for 2d. SCH 1 (7 mg) was dissolved in 1 ml DMSO, sterilfiltrated and added to the growing *A. oryzae* culture. After another 3d cultures were extracted and analysed as described above. *Aspergillus oryzae* NSAR1 WT fed with pure DMSO was used as a control experiment.

1.12. Transformation of *Aspergillus oryzae* NSAR1

A PEG-mediated transformation protocol was used for the ectopic integration of DNA into *Aspergillus oryzae* NSAR1. For this, conidia from a sporulating plate was inoculated into 100 ml GN medium (250 ml baffled flask) and kept at 30 °C, 180 rpm overnight. Mycelia was collected by filtration through sterile Miracloth and incubated in 10 ml protoplasting solution (20 mg ml$^{-1}$ trichoderma lysing enzyme; sterilized by filtration through a 0.45 µm syringe filter) at 30 °C and 70 rpm for 3h. Gentle pipetting with a wide-bore pipette released the protoplasts from the hyphal strains. Protoplasts were collected by centrifugation (10 min, 3000 x g, 4 °C) and resuspended in solution 1 (100 µl per transformation; 50 mM Tris-HCl pH 7.5; 800 mM NaCl; 10 mM CaCl$_2$). After addition of 10 µl purified plasmid DNA the mixture was incubated on ice for 2 min prior to addition of 1 ml solution 2 (60% w/v PEG3350, 50 mM Tris HCl pH 7.5, 800 mM NaCl, 10 mM CaCl$_2$). Additional incubation at room temperature for 20 min was followed by mixing with pre-warmed 5 ml CZD/S(1) softagar (-methionine) (50 °C) and overlayed onto pre-prepared CZD/S(1) plates (-methionine). Suitable agar was chosen according to auxotrophic resistance markers on the transformed plasmid DNA. All primary plates were kept at 28 °C for 3-5 days until visible formation of colonies. All colonies were subjected to two rounds of selection on arginine- (adenine or methionine) deficient media, depending on the nature of auxotrophic markers present on transformed plasmids. Prior to fermentation single colonies were grown on DPY agar for 1 week. For every colony, gDNA was isolated and ectopic integration of DNA was checked by PCR using gene specific primers (identical to those used for plasmid construction).
Figure S1 Biomimetic synthesis of SCH 1 by a key transannular Michael reaction reported by Snider and Zhou. (6)
Figure S2  Proposed mechanisms for SCH 1 biosynthesis based on Co-isolation of metabolites from Phomopsis sp. CMU-LMA.(7)
Figure S3  

A, BGC for Brefeldin A from *P. brefeldianum*. 

B, chain-length control of the hrPKS product of brefeldin A biosynthesis.\(^8\)
Figure S4

$^{13}$C-NMR of labelled SCH 1 (top) in CDCl$_3$ (125 MHz) compared to $^{13}$C-NMR of natural abundance SCH 1 (bottom).
Figure S5 Construction of KO cassettes for targeted gene disruption in *Phomopsis* sp. CMU-LMA.
Figure S6  PCR validation of gene disruption in Phomopsis sp. CMU-LMA. A, whole PCR; B, ext PCR.

Comment [CS]: Ext. PCR data for PKS, schR1, schR2 missing. Do we need this?
**Figure S7** $^1$H-NMR of compound 14 in CDCl$_3$ (500 MHz).
Figure S8: $^{13}$C-NMR of compound 14 in CDCl$_3$ (500 MHz).
Figure S9  COSY-NMR of compound 14 in Acetone-\textit{d}_6 (500 MHz).
Figure S10

Figure S10  HSQC-NMR of compound 14 in Acetone-d₆ (500 MHz).
Figure S11  H-4 decoupling in $^1$H-NMR for compound 14 and determination of key coupling constants. A and B are identical with B being the enlarged region between 5.40 and 5.65 ppm of A.
Figure S12  H-7/H-10 decoupling in $^1$H-NMR for compound 14 and determination of key coupling constants.
Figure S13  $^1$H-NMR of compound 16 in CDCl$_3$ (500 MHz).
Figure S14

$^{13}$C-NMR of compound 16 in CDCl$_3$ (500 MHz).
Figure S15  HSQC-NMR of compound 16 in CDCl$_3$ (500 MHz).
Figure S16  COSY-NMR of compound 16 in CDCl$_3$ (500 MHz).
Figure S17  HMBC-NMR of compound 16 in CDCl$_3$ (500 MHz)
Figure S18  Mass spectra (ES\textsuperscript{+}) and UV chromatograms of 1, 17, 18.
Figure S19  \(^1\)H-NMR of compound 17 in CDCl\(_3\) (500 MHz).
Figure S20  $^{13}$C-NMR of compound 17 in CDCl$_3$ (500 MHz).
Figure S21  HSQC-NMR of compound 17 in CDCl$_3$ (500 MHz).
Figure S22 COSY-NMR of compound 17 in CDCl$_3$ (500 MHz).
Figure S23  HMBC-NMR of compound 17 in CDCl₃ (500 MHz)
**Figure S24**

**19, t_R = 4.9 min**

- [M-2H_2O+H]^+ at 219.3 m/z
- [M-3H_2O+H]^+ at 201.3 m/z
- [M-H_2O+H]^+ at 237.3 m/z
- [M+H]^+ at 255.3 m/z

**20, t_R = 5.1 min**

- [M-2H_2O+H]^+ at 221.3 m/z
- [M-H_2O+H]^+ at 209.2 m/z
- [M+Na]^+ at 235.4 m/z
- [M-H]^+ at 271.3 m/z

**Figure S24** Mass spectra (ES^+ and ES^-) and UV chromatograms of 19 and 20.
Figure S25

$\text{H-NMR of compound 19 in CDCl}_3 (400 \text{ MHz})$
Figure S26

\[ ^{13}\text{C-NMR of compound 19 in CDCl}_3 (100 MHz) \]
Figure S27

**HSQC-NMR of compound 19 in CDCl₃ (400 MHz)**
Figure S28 COSY-NMR of compound 19 in CDCl$_3$ (400 MHz).
Figure S29

Figure S29  HMBC-NMR of compound 19 in CDCl$_3$ (400 MHz).
Figure S30

Thin-layer-chromatography (TLC) plate with one drop of each \textbf{16} and \textbf{19}, stained with pH indicator Bromocresol green – a color change to yellow indicating an acid.
Figure S31  Decoupling of H-7 in compound 19 $^1$H-NMR (500 MHz)
Figure S32  Decoupling of H-7 and H-10 in compound 19 \textsuperscript{1}H-NMR (600 MHz)
Figure S33 $^1$H-NMR of compound 20 in DMSO-$d_6$ (500 MHz).
Figure S34  $^{13}$C-NMR of compound 20 in DMSO-d$_6$ (500 MHz).
Figure S35  HSQC-NMR of compound 20 in DMSO-d$_6$ (500 MHz).
Figure S36  COSY-NMR of compound 20 in DMSO-d$_6$ (500 MHz).
Figure S37  HMBC-NMR of compound 20 in DMSO-d$_6$ (500 MHz).
Figure S38

A

Chemical analysis of extracts from *A. oryzae* NSAR1 transformed with *schPKS* and *schPKS + schR1* compared to *Aspergillus oryzae* NSAR1 wt control. **A**, coexpression of *schPKS* and *schR1*; **B**, DAD traces; **C**, ES+ traces.

**Figure S38** Chemical analysis of extracts from *A. oryzae* NSAR1 transformed with *schPKS* and *schPKS + schR1* compared to *Aspergillus oryzae* NSAR1 wt control. **A**, coexpression of *schPKS* and *schR1*; **B**, DAD traces; **C**, ES+ traces.
Figure S39

A, transformed genes; B, DAD traces; C, ES* traces

Figure S39  Chemical analysis of extracts from A. oryzae NSAR1 transformed with full sch BGC omitting only schR1 and compared to Aspergillus oryzae NSAR1 wt control. A, transformed genes; B, DAD traces; C, ES* traces
Figure S40  Cloning strategy schPKS.
Overview of generated expression plasmids for transformation of *A. oryzae* NSAR1.

*Figure S41*
3. Supplementary Tables

Table S1

| Description                             | Value                  |
|-----------------------------------------|------------------------|
| Alligned reads (all/paired ends)        | 54377401/23842462      |
| Assembled bases                         | 4201735013             |
| PE-Size(s)                              | 883±239; 884±239       |
| Scaffolds (all/true)                    | 2042/2042              |
| Contigs (Scaffolded/Large (>500 Bp)/all)| 5742/7578/28816        |
| Bases in Scaffolds                      | 64382824               |
| Coverage                                | 65.26 x                |
| GC content (%)                          | 52.92                  |
| Avg. Scaffold (Bp)                      | 31529                  |
| N\(_{50}\) Scaffold                     | 69395                  |
| Largest Scaffold                        | 420401                 |
| Avg. Scaffold Contig                    | 10656                  |
| Avg. Contig                             | 8321                   |
| N\(_{50}\) Contig                       | 19566                  |
| Largest Contig                          | 119136                 |

Table S1  Assembly details of *Phomopsis* sp. CMU-LMA WT.
Table S2

| BGC type          | Number of predicted BGC |
|-------------------|-------------------------|
| PKS               | 60                      |
| Terpene           | 26                      |
| Fatty Acid        | 5                       |
| NRPS              | 17                      |
| PKS-Terpene       | 3                       |
| Linaidin          | 1                       |
| Lantipeptide      | 1                       |
| Siderophore       | 1                       |
| Type III PKS      | 1                       |
| PKS-NRPS          | 19                      |
| PKS-NRPS-Terpene  | 1                       |
| Other             | 21                      |
| **Total**         | **156**                 |

Table S2  AntiSMASH prediction of secondary metabolites BGC in *Phomopsis* sp. CMU-LMA.
Table S3

| Position | CDCl₃ | Acetone-d₆ |
|----------|-------|-----------|
| CO₂      | 166.1 | 166.1     |
| CH=CH    | 118.2 | 117.7     |
| CH=CH    | 148.2 | 151.3     |
| CH-OH    | 68.5  | 68.5      |
| CH=CH    | 132.0 | 132.9     |
| CH=CH    | 131.2 | 132.8     |
| CH-OH    | 70.6  | 70.5      |
| CH=CH    | 134.4 | 133.0     |
| CH=CH    | 130.2 | 132.7     |
| CH₂      | 32.8  | 33.6      |
| CH₂      | 24.4  | 25.5      |
| CH₂      | 34.5  | 35.2      |
| CH-OH    | 72.05 | 72.3      |
| CH₃      | 20.2  | 20.6      |

Table S3  NMR data compound 14 (CDCl₃, 500 MHz).
### Table S4

| Position | $\delta_C$ / ppm | $\delta_H$ / ppm |
|----------|------------------|------------------|
| CO$_2$   | 1                | 166.3            | -                |
| CH=CH    | 2                | 117.5            | 5.94 (dd, 15.7, 1.9) |
| CH=CH    | 3                | 149.6            | 7.18 (dd, 15.7, 3.9) |
| CH-OH    | 4                | 68.3             | 5.26 (m)         |
| CH=CH    | 5                | 131.5            | 5.51 (m)         |
| CH$_3$   | 6                | 128.6            | 5.54 (m)         |
| CH=CH    | 7                | 32.1             | 2.82 (m)         |
| CH=CH    | 8                | 132.1            | 5.55 (m)         |
| CH=CH    | 9                | 126.6            | 5.43 (m)         |
| CH$_2$   | 10a              | 32.9             | 2.08 (m)         |
|          | 10b              |                  | 1.91 (m)         |
| CH$_3$   | 11a              | 24.4             | 1.78 (m)         |
|          | 11b              |                  | 1.19 (m)         |
| CH$_2$   | 12a              | 34.3             | 1.74 (m)         |
|          | 12b              |                  | 1.51 (m)         |
| CH-OH    | 13               | 71.8             | 4.82 (m)         |
| CH$_3$   | 14               | 20.1             | 1.27 (d, 6.4)    |

*Table S4*  NMR data compound 16 (CDCl$_3$, 500 MHz).
Table S5

Table S5  Chemical shifts of 1.2 mg 17 in CDCl₃. Referenced to Adelin et al.(9)

| Atom | δ_C / ppm | δ_H / ppm | J / Hz   | δ_C / ppm | δ_H / ppm | J / Hz   |
|------|-----------|------------|----------|-----------|------------|----------|
| 1    | 210.5     | -          | -        | 211       | -          | -        |
| 2a   | 35.8      | 2.24       | dddd (14.3, 4.8, 2.4, 0.7) | 35.8 | 2.2 | m |
| 2b   | 35.8      | 2.6        | ddt (14.3, 6.4, 0.7) | 35.8 | 2.66 | m |
| 3a   | 32.5      | 1.85       | ddd (14.1, 4.8, 2.2) | 32.5 | 1.78 | m |
| 3b   | 32.5      | 2.15       | dddd (14.0, 6.1, 3.5, 2.3) | 32.5 | 1.94 | m |
| 4    | 71.0      | 4.13       | q (2.7)  | 70.9      | 4.1        | m |
| 5    | 41.8      | 2.37       | dtd (12.1, 6.6, 2.2) | 41.7 | 2.36 | q (7.3) |
| 6    | 47.0      | 2.66       | m        | 47.8      | 2.66       | m |
| 7    | 22.1      | 1.47       | m        | 21.2      | 1.35       | m |
| 8    | 22.7      | 1.20, 1.76 | m        | 22.2      | 1.35       | m |
| 9    | 25.2      | 1.40, 1.60 | m        | 22.7      | 1.35       | m |
| 10   | 33.2      | 1.35       | m        | 33        | 1.35       | m |
| 11   | 74.1      | 5.1        | m        | 74.1      | 5.07       | m |
| 12   | 173.2     | -          | -        | 173.2     | -          | - |
| 13   | 39.7      | 2.60, 2.76 | m, d (5.4) | 39.8 | 2.66 | m |
| 14   | 19.7      | 1.26       | d (6.4)  | 19.7      | 1.23       | d (6.4) |
Table S6

| Atom | $\delta_C$ / ppm | $\delta_H$ / ppm | J / Hz | $^1$H-$^1$H COSY | HMBC |
|------|------------------|------------------|--------|------------------|------|
| 1    | 167.4            | -                | -      | -                | 2, 3 |
| 2    | 120.0            | 5.86             | dd, 15.4, 1.6 | 3       | 1, 3, 4 |
| 3    | 149.1            | 6.67             | dd, 15.4, 4.9 | 2, 4   | 1, 2, 4, 5 |
| 4    | 65.9             | 4.99             | d, 6.2 | 3, 5             | 2, 3, 5, 6 |
| 5    | 131.0            | 5.29             | m      | 6                | 3, 4, 6, 7 |
| 6    | 128.8            | 5.42             | m      | 5, 7             | 4, 5, 7 |
| 7    | 30.4             | 2.79             | m      | 6, 8             | 5, 6, 8, 9 |
| 8    | 130.9            | 5.39             | m      | 7, 9, 10         | 6, 7, 9 |
| 9    | 127.5            | 5.42             | m      | 10               | 10, 11 |
| 10   | 32.1             | 1.95             | m      | 9, 11a, 11b, 12  | 9, 11 |
| 11a  | 25.3             | 1.36             | m      | 10, 12           | 9, 10 |
| 11b  | 25.3             | 1.28             | m      | 10, 12, 13       | 9, 10 |
| 12   | 38.6             | 1.28             | m      | 10, 11           | 13, 14 |
| 13   | 65.6             | 3.56             | m      | 12, 14           | 10, 11, 12, 14 |
| 14   | 23.7             | 1.02             | d, 6.1 | 13               | 12, 13 |

Table S6  Chemical shifts of 1.2 mg 19 in CDCl$_3$. Referenced to Adelin et al.(9)
Table S7

| Atom | δ_c / ppm | δ_δ / ppm | J / Hz | 1H-1H COSY | HMBC |
|------|------------|------------|--------|-------------|------|
| 1    | 167.2      | -          | -      | -           | 2, 3 |
| 2    | 119.6      | 5.86       | dd, 15.6, 1.6 | 3         | 1, 3, 4 |
| 3    | 149.6      | 6.67       | dd, 15.6, 4.9 | 2, 4      | 1, 2, 4, 5 |
| 4    | 65.8       | 4.99       | m      | 3, 5        | 2, 3, 5, 6 |
| 5    | 131.0      | 5.28       | ddt, 10.9, 8.2, 1.5 | 4, 6    | 3, 6, 7 |
| 6    | 128.7      | 5.44       | m      | 5, 7        | 4, 5, 7 |
| 7    | 30.4       | 2.79       | m      | 6           | 6, 8, 9 |
| 8    | 130.2      | 5.39       | m      | 7, 9        | 7, 9 |
| 9    | 128.1      | 5.39       | m      | 8, 10       | 7, 8, 10 |
| 10   | 30.3       | 1.93       | m      | 9, 11       | 8, 9, 10, 11 |
| 11   | 23.0       | 1.50       | p, 7.4 | 10, 12      | 9, 10, 12, 13 |
| 12   | 42.0       | 2.39       | t, 7.3 | 11          | 10, 11, 13 |
| 13   | 208.3      | -          | -      | -           | 11, 12, 13 |
| 14   | 29.7       | 2.06       | S      | -           | 12, 13 |

Table S7 Chemical shifts of 1.9 mg 20 in DMSO.
Table S8

| Primers used for the construction of KO plasmids |
|-----------------------------------------------|
| schPKS TL Fw | GCCAACTTTTGTAACAAAAAGCAGGCTCCGCATGCCTTCGTACATACATA |
| schPKS TL Re | AAACGAAATGGGCAGAACACAT |
| schPKS HygR Fw | CCTCTTTTTACGTTGTGTTGCTTTTCATTTCTAGGGATCTTTTGACAC |
| schPKS TR Fw | CCCGAAGCTCTGAGGGGCAAGGAATAGGGGACCTGGCCAGAAAGGAAG |
| schPKS TR Re | TGCCAACTTTGTACAAAGAAGCTGGTGCTGACGTGATACAATCTCTTG |
| schR2 TL Fw | GCCAATCTTTGTCACAAAAAGCAGGCTCCGCAACCGGACATCTCGACACTC |
| schR2 TL Re | GGAACCGGACCTCCCGACGCT |
| schR2 HygR Fw | AGCTATGGTGACAGACTGAAAGTGGAGTTCTCTATGGAATTTTGACAC |
| schR2 TR Fw | CCCGAGCACTCTGAGGGGCAAGGAATAGGACCCAGAATGAATCTTCGGC |
| schR2 TR Re | TGCCAACTTTGTACAAAGAAGCTGGTGCTGACGTGATACAATCTCTTG |
| schR3 TL Fw | GCCAACCTTTGTACAAAAAGCAGGCTCCGCAATATTCATGCTCTTGGGC |
| schR3 TL Re | AATCAGGGTATTCGAGAAAG |
| schR3 HygR Fw | TATCATATGTACCTTCTCAATACCTGATTTCTAGGGATCTTTTGACAC |
| schR3 TR Fw | CCCGAGCACTCTGAGGGGCAAGGAATAGGACCCGTCAGAATCTTCGGC |
| schR3 TR Re | TGCCAACTTTGTACAAAGAAGCTGGTGCTGACGTGATACAATCTCTTG |
| schR4 TL Fw | GCCAATCTTTGTCACAAAAAGCAGGCTCCGCAATGATCTACCTGGGGAG |
| schR4 TL Re | CAAGCTTGCCATGGGTCAGATAC |
| schR4 HygR Fw | CAGCTTCCATGATACTGCACCATAGCTGATCTTCTTCTGCTCCGAG |
| schR4 TR Fw | ACGTATTTGACGATGCAAGAATAGGACCCAGAATGAATCTTCGGC |
| schR4 TR Re | TGCCAACTTTGTACAAAGAAGCTGGTGCTGACGTGATACAATCTCTTG |
| schR5 TL Fw | GCCAATCTTTGTCACAAAAAGCAGGCTCCGCAACCGGACATCTCGACACTC |
| schR5 TL Re | GTCAGTTGGGAAATGCCAAG |
| schR5 HygR Fw | CCTGAAGGAACAGTGGAACATTCCAGTGACTATGGGATCTTTTGACAC |
| schR5 TR Fw | CCCGAGCACTCTGAGGGGCAAGGAATAGGACCCGTCAGAATCTTCGGC |
| schR5 TR Re | TGCCAACTTTGTACAAAGAAGCTGGTGCTGACGTGATACAATCTCTTG |
| schR7 TL Fw | GCCAATCTTTGTCACAAAAAGCAGGCTCCGCAACCGGACATCTCGACACTC |
| schR7 TL Re | GTAGAGGTGGGTCAG |
| schR7 HygR Fw | GCATCTCCGACTGACGAGGAGACCTGACTGATGGGATCTTTTGACAC |
| schR7 TR Fw | CCCGAGCACTCTGAGGGGCAAGGAATAGGACCCAGAATGAATCTTCGGC |
| schR7 TR Re | TGCCAACTTTGTACAAAGAAGCTGGTGCTGACGTGATACAATCTCTTG |

Table S8 Primers used for the construction of KO plasmids
Table S9

| Primers used for external PCR in KO validation |
|-----------------------------------------------|
| HygRP7.2 Fw | GCTTTCAAGCTCGATGTAGG |
| HygRP6.2 Re | CGTCAGGACATTGTTGGAG |
| schPKS ext  | CTAGGCCTAGACTACTTGAC |
| schR2 ext   | GAGAAAGGCGATTGAGTC |
| schR3 ext   | GAATCAAAAGCCACCGTAATG |
| schR5 ext   | GCATTAGCTCAACGGACC |
| schR7 ext   | GCATTAGCTCAACGGACC |
### Table S10

| Construct ID | Vector backbone | Template | Oligonucleotides for construction in *S. cervisiae* |
|--------------|-----------------|----------|---------------------------------------------------|
| KELII02A(No2_3)* | pE–YA | cDNA | schPKS 4 fragments: P966-P973 |
| KELII07* | pE–YA | KELII02A(No2_3) | schPKS 7 fragments: P966+1103, P1104+1109, P1110+1079, P1080+1081, P1082+1083, P1084+1085, P1086+973 |
| KELII08 | pE–YA | KELII02A(No2_3) | Patch: P1104+1116; KELII07 cut with KpnI |
| KELII02B | pTYGSarg | cDNA | schR1: P975+976 |
| KELII02C* | pTYGsmet | cDNA | schR1: P975+977, schR2: P978+979, schR3*: P980+981 |
| KELII02D | pTYGsmet | cDNA | schR4: P982+983, schR5: P984+985, schR7: P986+987 |
| KELII08A | pTYGSarg | - | LR with KELII08 and KELII02A |
| KELII08B | pTYGSarg | - | LR with KELII08 and KELII02B |
| KELI102C* | pTYGSarg | - | LR with KELII08 and KELII02C |
| KELI10B | pTYGsad | KELII08C, gDNA (schR3) | schR1: P975+977, schR2: P978+979, schR3: P980+981 |
| KELI10C | pTYGsad | KELII08C | schR1: P975+977, Patch_Peno: P87+88 |
| KELI10A | pTYGsmet | KELII02D | Patch_Padh: P89+90, Patch_PgdpA: P91+92, schR3: P980+981 |
| KELI11B | pTYGsmet | KELII02D | Patch_Padh: P89+90, schR5: P984+985, schR7: P986+987 |
| KELI11C | pTYGsmet | KELII02D | schR4: P982+983, Patch_PgdpA: P91+92, schR7: P986+987 |
| KELI11D | pTYGsad | KELII010A | Patch_Padh: P89+90, schR2: P978+979, schR3: P980+981 |

*point mutations in gene

**Table S10** Construction details for fungal transformation plasmids used for heterologous expression of SCH genes in *A. oryzae*
|   | Oligonucleotides used in the construction of fungal expression vectors |
|---|---------------------------------------------------------------------|
| 87| CTTCTAAATATCCTCCTTGAAACGTCCTGGAATACCCATGCTCCTGGA |
| 88| CGAATATATTGGGAGACTATAGCTACCTAG |
| 89| CGAATATATTGGGAGACTATAGCTACCTAG |
| 90| GAGACGAAACAGACTTTTTCATCCGGATGTTAGCCCTGCTCATTACGATACAGAA |
| 91| CTTCTAAATATCCTCCTTGAAACGTCCTGGAATACCCATGCTCCTGGA |
| 92| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 93| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 94| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 95| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 96| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 97| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 98| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 99| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 100| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 101| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 102| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 103| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 104| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 105| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 106| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 107| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 108| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 109| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 110| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 111| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |
| 112| GCCAACCTTTTCATCACAAAAACGAGGCCTGCGCATGCTCGTCTGACATAACAAC |

*Table S11* Oligonucleotides used in the construction of fungal expression vectors
4. Supplementary references

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