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Supporting Information

Biosynthesis of Sinapigladioside, an Antifungal Isothiocyanate from *Burkholderia* Symbionts

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Experimental Procedures

Bacterial strains, media and cultivation conditions

Burkholderia gladioli HKI 0739 (syn. *Burkholderia gladioli* Lv-StA) was cultured in liquid MGY medium (yeast extract: 1.25 g L^{-1}, glycerol: 10 g L^{-1}, M9 salts, part A: 70 g L^{-1} K_2HPO_4, 20 g L^{-1} KH_2PO_4; part B: 0.58 g L^{-1} tri-sodium citrate dihydrate, 1 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} $MgSO_4$), or in potato dextrose broth (PDB, Bacto®; potato starch 4.0 g L^{-1}, dextrose 20.0 g L^{-1}) at 30 °C and 110 rpm orbital shaking, or on solid media PDA (add 15 g L^{-1} agar to PDB) or NAG (standard I nutrient agar, Merck®; peptone 15 g L^{-1}, yeast extract 3 g L^{-1}, sodium chloride 6 g L^{-1}, glucose1 g L^{-1}, agar 12 g L^{-1}, glycerol 10 g L^{-1}, pH value 7,5 ± 0,2). Sinapigladioside (**9**) production peaks after cultivation for 24 h in PDB. For long-term storage bacterial cells were frozen in 1:1 *v/v* of 50% glycerol at –80 °C. For genetic manipulations *B. gladioli* HKI0739 was cultured in MGY + M9 or PDB medium/agar with addition of kanamycin (100 µg mL^{-1}).
E. coli strains were cultured in LB medium (10 g tryptone (BD, Bacto®), 5 g yeast extract (BD, Bacto®), 10 g NaCl, sterilization at 120 °C for 20 min; for agar: addition of 1.5% agar) or on LB agar plates at 37 °C with appropriate antibiotic concentrations (kanamycin 50 µg mL^{-1}, gentamicin 20 µg mL^{-1}).

General analytical methods

Analytical LC-MS system: Exactive Orbitrap High Performance Benchtop LC-MS (Thermo Fisher Scientific, Germany) with an electron spray ion source and an Accela HPLC System, C18 column (Betasil C18, 150 × 2.1 mm, Thermo Fisher Scientific, Germany), solvents: acetonitrile and distilled water (both supplemented with 0.1% formic acid), flow rate: 0.2 mL min^{-1}; program: hold 1 min at 5% acetonitrile, 1–16 min 5–99% acetonitrile, hold 16–31 min 99% acetonitrile, 31–32 min 99–5%, 32–43 min to 5% acetonitrile.

MS-MS: QExactive Orbitrap High Performance Benchtop LC-MS (Thermo Fisher Scientific, Germany) with an electron spray ion source and an Accela HPLC System, C18 column (Accucore C18 2.6 µm, 100 × 2.1 mm, Thermo Fisher Scientific, Germany), solvents: acetonitrile and distilled water (both supplemented with 0.1% formic acid), flow rate: 0.2 mL min^{-1}; program: hold 1 min at 5% acetonitrile, 1–16 min 5–99% acetonitrile, hold 16–31 min 99% acetonitrile, 31–32 min 99–5%, 32–43 min to 5% acetonitrile.

NMR spectra were recorded with a (Bruker 500 bzw. 600 MHz Avance III Ultra Shield (Bruker BioSpin GmbH, Rheinstetten, Deutschland) in DMSO-d_6.

Optical rotation: Jasco P-1020 polarimeter, Na light (589 nm), at 25 °C, 50 mm cell length, *c* 2 *w*/*v*%, dissolved in 83% acetonitrile (83% MeCN).

Isolation of sinapigladioside (9) and biosynthetic intermediate 13

To isolate sinapigladioside (**9**) 3 L of PDB were inoculated and incubated at 30 °C and 110 rpm for 24 h.[1] The cultures were extracted with ethylacetate (1 : 1) and concentrated under reduced pressure. The dry extract was separated by size-exclusion chromatography using a Sephadex LH-20 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and 83% MeCN as an eluent. The metabolite-containing fraction was further purified by preparative HPLC using a C18 column (Nucleodur VP, 250 × 21 mm, C18 HTec, 5 µm) and a solvent system of solvent A, (H_2O + 0.01% trifluoroacetic acid) and solvent B (83% MeCN) with a flow rate of 10 mL min^{-1} and a gradient method (acetonitrile/0.01 trifluoroacetic acid (H_2O, *v*/*v*) 30/70 for 5 min and subsequently to 100/0 in 20 min).

Annotation of sinapigladioside biosynthetic gene cluster (*spg*)

The *spg* biosynthetic gene cluster (Figure S1) was annotated based on homology to characterised genes (Table S1). The genome sequence of *Burkholderia gladioli* HKI0739 (syn. *Burkholderia gladioli* Lv-StA) can be found under the following entry in the GenBank: WITE01000001.1.

Figure S1. Sinapigladioside biosynthetic gene cluster (*spg*) from *B. gladioli* HKI0739.

Table S1. Genes encoded in the sinapigladioside biosynthetic gene cluster (*spg*).

Genes	Size [bp]	Characterised homologous proteins, Sequence ID [Identity/Similarity]	Species	Proposed function of encoded protein
spgA	1,008	Isocyanide synthase XanB, Q4WED9.2 [25%/40%]	*Aspergillus fumigatus* Af293	Isonitrile synthase A
spgB	1,206	Inner membrane transport protein YdhC, P37597.3 [34%/51%]	*E. coli* K-12	Self-resistance mechanism
spgC	471	Hypothetical protein	/	/
spgD	1,833	*Isn*A-*Isn*B fusion gene N-Terminal: Isocyanide synthase-NRPS hybrid, crmA3E59_A [26%/39%] C-Terminal: Isocyanide synthase XanB, Q4WED9.2 [25%/40%]	*Aspergillus fumigatus* Af293	Isonitrile synthase B
spgE	840	PGL/p-HBAD biosynthesis glycosyltransferase, A5U6W6.1 [40%/53%]	*Mycobacterium tuberculosis* H37Ra	Sugar transfer
spgF	2,431	Hypothetical protein	/	/
spgG	678	*S*-Adenosyl-L-methionine-dependent methyltransferase, 4HTF_A [19%/17%]	*Escherichia coli* O157:H7	Transfer of a methyl group
spgH	1,419	Decaprenyl-phosphate phosphoribosyltransferase, P9WFR4.1 [36%/53%]	*Mycobacterium tuberculosis* CDC1551	Unknown function
spgI	1,191	Decaprenylphosphoryl-beta-D-ribose oxidase, P9WJF0.1 [37%/49%]	*Mycobacterium tuberculosis* CDC1551	Unknown
spgJ	741	Rhamnulose-1-phosphate aldolase/alcohol dehydrogenase cd08943 [13%/18%]	Template alignment; rhamnulose-1-phosphate aldolase/alcohol dehydrogenas	Putative xylose biosynthesis
spgK	714	UDP-Glucuronate decarboxylase cd05230 [1%/17%]	Template alignment; UDP-glucuronate decarboxylase and related proteins	Putative xylose biosynthesis
spgL	1,296	UDP-Galactopyranose mutase, 4U8I_A [15%/18%]	*Aspergillus fumigatus*	Putative xylose biosynthesis
spgY	1,008	HTH-Type transcriptional regulator CdhR, Q9HTH5 [29%/47%]	*Pseudomonas aeruginosa* PAO1	Regulation
spgZ	1,062	Hypothetical protein	/	/

Proteins encoded in the genome of *Burkholderia gladioli* HKI0739 that contain a rhodanese homology domain (RHOD) and thus are potential candidates for the sulfur transfer in the biosynthesis of **9** are listed in .

Table S2.

Table S2. Proteins containing a RHOD.

Accession number	Size [AA]	Name
KAF1058013.1	289	Putative thiosulfate sulfurtransferase; contains two RHOD
KAF1058191.1	552	Thiosulfate sulfurtransferase; contains four RHOD Repeat
WP_036036664.1	130	Rhodanese; contains one RHOD
KAF1063262.1	156	Putative protein YibN; contains one RHOD
KAF1064393.1	109	Sulfurtransferase; contains one RHOD

Multiple sequence alignment of isonitrile synthase sequences

Since isonitrile synthases (IsnA) involved in the biosynthesis of various isonitrile containing secondary metabolites have been characterised, the active site residues respectively the conserved domains are known.[2] Using a multiple sequence analysis of these characterised IsnA homologues and the putative IsnA from *B. gladioli* HKI0739 six conserved sequence motifs (I–VI) were found (Figure S2). The sequences were aligned using T-Coffee,[3] and the representation was prepared with BOXSHADE.[4]

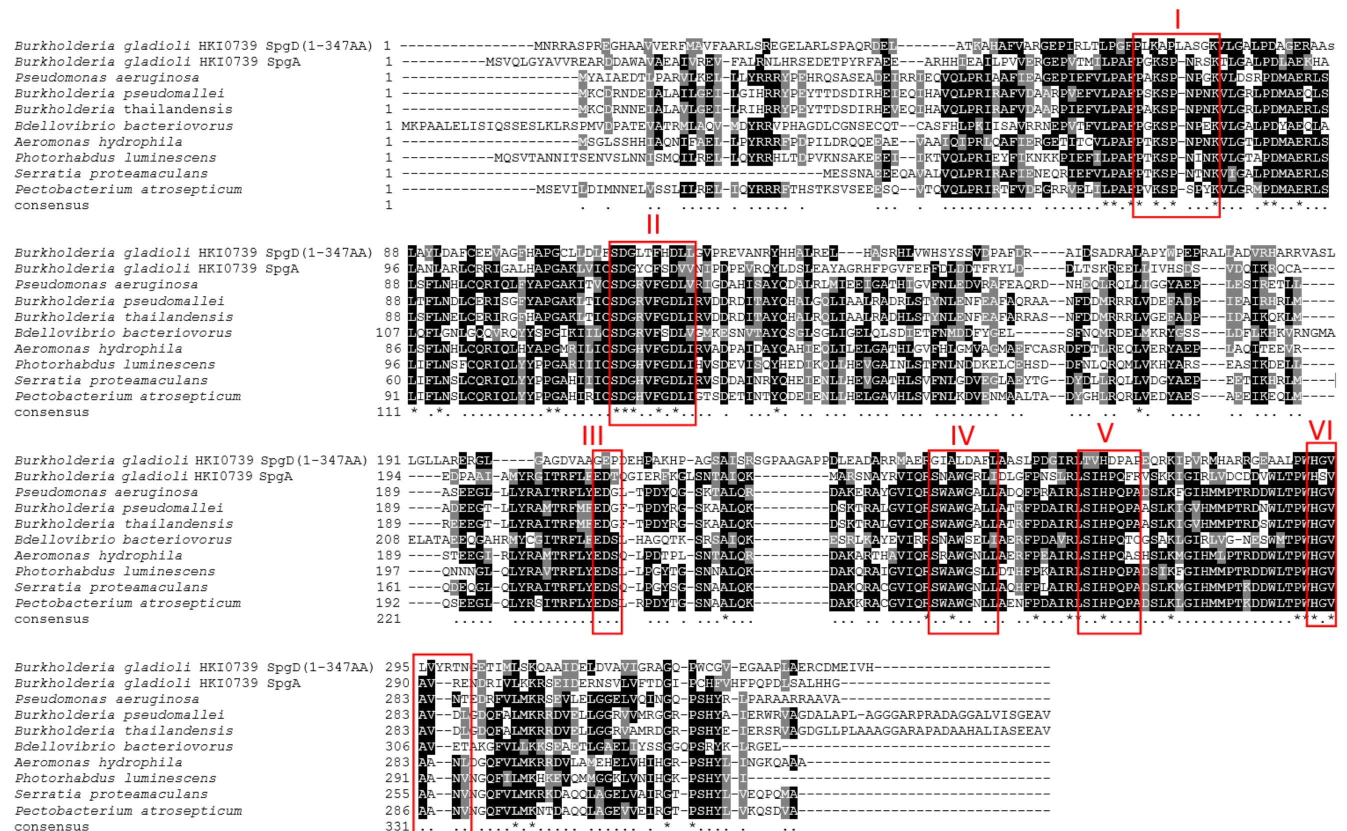


Figure S2. Multiple sequence alignment of IsnA homologues, including SpgA. Six conserved motifs are identified and marked with red boxes. Full sequence information: *Burkholderia gladioli* HKI0739 (*spgA*; WP_052747423.1) and (*spgD*; WP_160292732.1), *Pseudomonas aeruginosa* (AAG05642.1), *B. pseudomallei* 668 (ABN87870.1), *B. thailandensis* E264 (ABC34526.1), *Bdellovibrio bacteriovorus* strain HD100 (CAE79340.1), *Aeromonas hydrophila* subsp. *hydrophila* (ABK38251.1), *Photorhabdus luminescens* subsp. *laumondii* TTO1 (CAE15190.1), *Serratia proteamaculans* 568 (ABV39809.1), *Pectobacterium atrosepticum* SCRI1043 (CAG76279.1).

Furthermore, an IsnB homologue (611 residues) is encoded in the proximity to the putative *isnA*-gene in the genome of *B. gladioli*. This gene appears to code for a fusion protein consisting of a C-terminal IsnA and an N-terminal IsnB domain. Potential IsnA-IsnB bifunctional proteins (~ 700 residues) have previously been found in some bacteria and fungi, although the presence of an additional IsnA protein was not reported.[5] An alignment of the N-terminal part of SpgD (amino acid 1 to 347), SpgA and other IsnA-proteins showed, that the amino acid sequence of SpgD differs from the conserved motifs that were previously identified in IsnA-proteins.[2] Thus, the N-terminal part of SpgD might be inactive (Figure S2). Note, that the active site amino acids residues of IsnA-proteins are unknown.

IsnB homologues are predicted to belong to the family of Fe^{2+}/α-KG-dependent oxygenases. The multiple alignments of the C-terminal part of IsnA-IsnB (beginning from amino acid 347) show the conservation of the active site residues (Figure S3). Accordingly, the functionality should be similar.[2] Members of this family of enzymes catalyze the incorporation of one atom of oxygen from molecular oxygen into a wide variety of products.[6] The other oxygen atom reacts with α-ketoglutarate to form succinate and CO_2. The Fe^{2+} ion is coordinated by three amino acid residues, two histidines and an aspartic or glutamic acid (Figure S3).

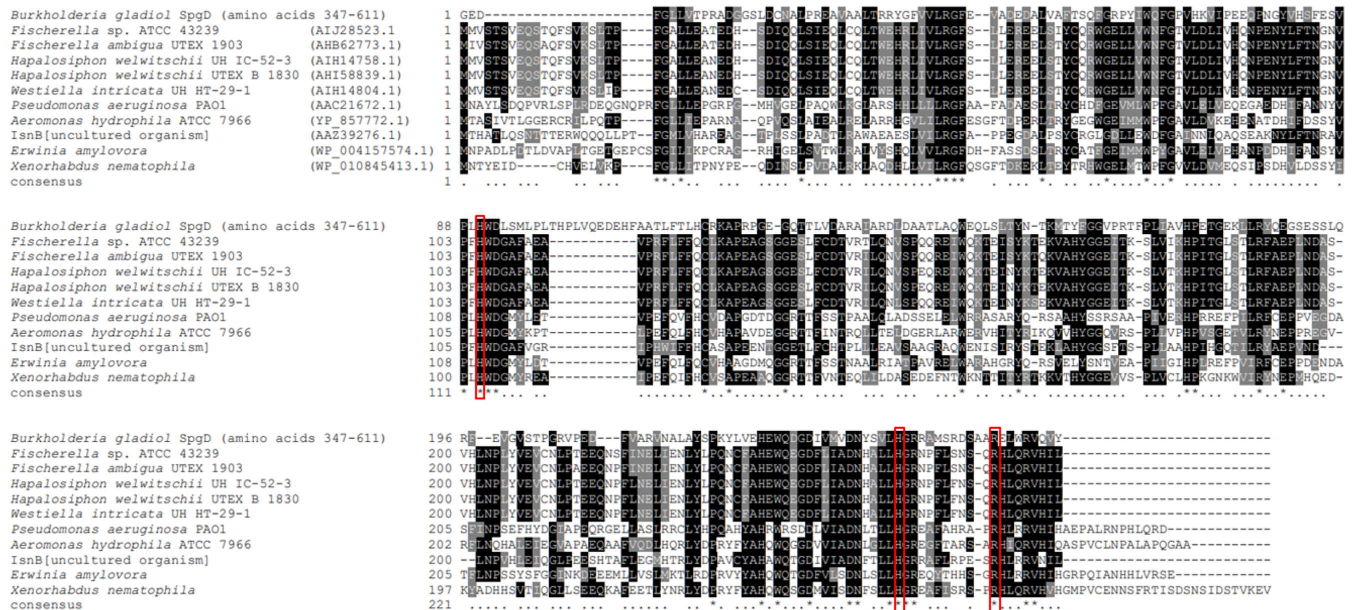


Figure S3. Multiple sequence alignment of the C-terminal IsnA-IsnB fusion protein SpgD (amino acids 347–611). Four previously identified amino acid residues that constitute the active site are marked with the red boxes.[2] Full sequence information: *B. gladioli* HKI0739 spgD (WP_160292732.1), *Fischerella* sp. (ATCC 43239) (AIJ28523.1), *Fischerella ambigua* UTEX 1903 (AHB62773.1), *Hapalosiphon welwitschii* UH IC-52-3 (AIH14758.1), *Hapalosiphon welwitschii* UTEX B 1830 (AHI58839.1), *Westiella intricata* UH HT-29-1 (AIH14804.1), *Pseudomonas aeruginosa* PAO1 (AAC21672.1), *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (YP_857772.1), uncultured organism IsnB (AAZ39276.1), *Erwinia amylovora* (WP_004157574.1), *Xenorhabdus nematophila* (WP_010845413.1).

Genetic manipulation of *Burkholderia gladioli* HKI0739

Genes of the sinapigladioside biosynthetic gene cluster were inactivated using suicide gene knockout vectors that were transferred into *B. gladioli* HKI0739 by electroporation. Upon homologous recombination (double-crossover) the gene of interest is inactivated due to the integration and partial deletion of the target gene by an antibiotic cassette (*kanR* or *apraR*). The resistance cassette enables subsequent selection of mutant strains. Homologous regions up- and downstream of the target genes were typically ~ 1,000 bp in size. The sizes vary as primers were created by the aid of Primer3[7] to optimize GC-content and binding in PCRs (default settings but GC-clamp: 1; Number of consecutive Gs and Cs at the 3' end of both the left and right primer). The successful integration and disruption of the target gene was verified by colony PCR and analysis of the metabolic profile of the mutant strain.

Cloning of gene knockout plasmids

All experiments have been performed according to manufacturer's recommendations if not stated otherwise. Genomic DNA was purified using the PureTM DNA-Isolation kit (Epicentre Biotechnologies). PCR reactions, if not stated otherwise, were performed using KAPA2G Robust HotStart ReadyMix PCR kit (Sigma-Aldrich) according to manufacturer's recommendations. PCR products and restriction mixtures were purified using either the innuPREP PCRpure Kit or the innuPREP Gel Extraction Kit (Analytik Jena, Germany). All restriction endonucleases were purchased from New England Biolabs, Frankfurt am Main, Germany. Two cloning strategies were used: NEBuilder based cloning or restriction and ligation cloning (Figure S4).

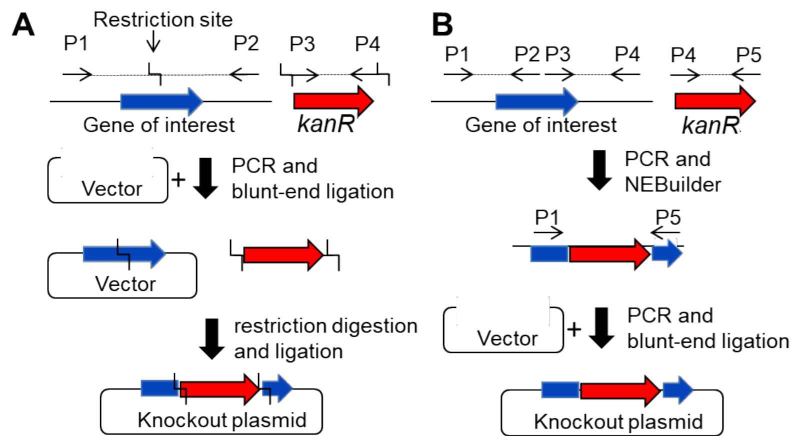


Figure S4. Cloning strategies to generate gene knockout plasmids. A) Cloning of gene knockout plasmids using a restriction and ligation approach or B) NEBuilder based cloning strategy. P, primer; *kanR*, kanamycin resistance cassette.

Construct pBD70 was constructed as follows: A PCR product generated using the primers BD332 and BD333 and genomic DNA from *B. gladioli* HKI0739, was blunt-end ligated into pJet1.2. The PCRs were performed using Phusion High-Fidelity DNA Polymerase with 5% DMSO (New England Biolabs, Frankfurt am Main, Germany) and 35 cycles (98 °C 15 s, 65 °C 15 s, 72 °C 1 min kb^{-1}). Top10 *E. coli* cells were transformed with the resulting plasmids. Positive colonies were detected using a colony PCR screening approach. After plasmid isolation using the Monarch® Plasmid Miniprep Kit (New England Biolabs, Frankfurt am Main, Germany). The plasmid was digested with *Pml*I. A kanamycin resistance cassette (*kanR*) was generated using the primer BD334 and BD335 and pET28_a. The product was subsequently digested with *Pml*I. A ligation of the two DNA fragments yielded pBD70.

All other constructs where cloned as follows: The flanking regions next to the gene of interest were generated using the primers and templates listed in Table S3. The PCRs were performed using Phusion High-Fidelity DNA Polymerase with 5% DMSO (New England Biolabs, Frankfurt am Main, Germany) and 35 cycles (98 °C 15 s, 65 °C 15 s, 72 °C 1 min kb^{-1}). Three PCR products per plasmid were subjected to a NEBuilder reaction according to the manufacture's recommendations. The NEBuilder mix was subsequently blunt-end ligated into pJET1.2 yielding the respective plasmids (Table S4).

Generation of electrocompetent *Burkholderia gladioli* HKI0739 cells

B. gladioli HKI0739 cells were grown in MGY + M9 medium and incubated at 30 °C and shaking at 110 rpm until cells reached OD_{600} of ~ 0.8. The cells were harvested by centrifugation at 6,000 × g at room temperature for 5 min and washed twice in the same volume of sucrose solution (300 mM in water). The resulting cell pellet was resuspended in sucrose solution (100 µL per 4–5 mL bacteria culture). Typically, four to five transformations can be performed from a 20 mL culture of *B. gladioli* HKI0739. Two µL of the gene knockout plasmids listed in Table S4 were added to 100 µL cell solution before by electroporation. Electroporations were performed at 2.5 kV in a 2 mm gapped electroporation cuvette. Subsequently, 500 µL of MGY + M9 medium was added to the cell solution. After incubation at 30 °C and with shaking at 100 rpm for 3–4 h, the cell solution was plated on NAG agar plates supplemented with either kanamycin (100 µg mL^{-1}) or apramycin (300 µg mL^{-1}) and incubated at 30 °C until colonies grew.

Confirmation of gene knockout strains

Homologous recombination of the plasmids with the corresponding region in the genome yielded the gene knockout strains. The target gene is disrupted and partially deleted by the resistance cassette. The successful integration was verified by colony PCR using primers listed in Table S5S3 and S5 and colony material with a PCR master mix of KAPA2G Robust HotStart ReadyMix PCR kit (Merck KGaA, Darmstadt, Germany) with 3% DMSO and 35 cycles of 95 °C for 30 s, 60 °C for 30 s kb^{-1}, and a final extension time at 72 °C for 300 s. The generation of PCR products of the sizes listed in Table S5 allows for differentiation between mutants and wild-type strains (Figure S5).

For verification of gene knockouts in *B. gladioli* pBD41–45 strains, PCRs amplifying the front (F) or back (B) regions of the target gene were performed. Only if homologous recombination occurred, a PCR product can be formed as one primer binds in the resistance cassette while the other binds in the genome of the bacteria. PCRs amplifying the deleted regions of the target genes were performed as controls (C). For verification of successful gene knockouts in *B. gladioli* pBD47–53 and pBD70, PCRs amplified the target gene using primers binding up- and down-stream. Thus, upon homologous recombination and integration of the resistance cassette the PCR-products of the mutant strains increase in sizes.

Table S3. Primers used in this study. *Bg*739, genomic DNA from *B. gladioli* HKI0739.

Primer	Sequence 3’ → 5’	Template	Product size [bp]	Purpose
BD103	actgttgcaaatagtggccgtctcccggcgtcg	*Bg*739	988	cloning of pBD41
BD104	cgccgggagacggccactatttgcaacagtgccgttg			
BD105	tccgatcgaatcggttcagccaatcgactggcg	pET28a	1,186	
BD106	cagtcgattggctgaaccgattcgatcggaatcctg			
BD107	taaggcgccgagcgtcttg	*Bg*739	782	
BD108	actgttgcaaatagtggccgtctcccggcgtcg			
BD154	tggaactgcagcatgaacag	*Bg*739	Table S5	colony PCR
BD155	aggtcaggtcgtcgaggtag			
BD109	aggagctggccgccgcgg	*Bg*739	1,015	cloning of pBD42
BD110	actgttgcaaatagtgatttctctcaggatggatggtcaacgccatggc			
BD111	atcctgagagaaatcactatttgcaacagtgccgttg	pIJ773	875	
BD112	ctcaggtccggttgctcagccaatcgactggcg			
BD113	cagtcgattggctgagcaaccggacctgagcgc	*Bg*739	1,015	
BD114	ccgaacggggcgacgtag			
BD156	ggacgatcgcatctacattg	*Bg*739	Table S5	colony PCR
BD157	gattccgaattgcttcatgc			
BD115	ctgaagcgggctcattcgacgc	*Bg*739	1,015	cloning of pBD43
BD116	actgttgcaaatagtagcgcgcgatccgcgctg			
BD117	cgcggatcgcgcgctactatttgcaacagtgccgttg	pIJ773	875	
BD118	cctgcaccagcggattcagccaatcgactggcg			
BD119	cagtcgattggctgaatccgctggtgcaggaggac	*Bg*739	1,015	
BD120	gcctgggcacgaacggcag			
BD158	gatcggcatcaccattcc	*Bg*739	Table S5	colony PCR
BD159	gaaccgagcgagacgtagac			
BD121	tgggacctgagcatgctg	*Bg*739	1,015	cloning of pBD44
BD122	actgttgcaaatagtgttcaggccgcggatctc			
BD123	atccgcggcctgaacactatttgcaacagtgccgttg	pIJ773	875	
BD124	ggcctcggtcgtgtttcagccaatcgactggcg			
BD125	cagtcgattggctgaaacacgaccgaggccggc	*Bg*739	1,015	
BD126	gggcagcaggtagagcgc			
BD160	ccgattgccagaacgaatag	*Bg*739	Table S5	colony PCR
BD161	cgtgtatcgaacgaatggag			
BD127	tccggtgatccagagctac	*Bg*739	1,000	cloning of pBD45
BD128	actgttgcaaatagtgtgcaggccggccgggtc			
BD129	ccggccggcctgcacactatttgcaacagtgccgttg	pIJ773	875	
BD130	ggccagcgcttcaggtcagccaatcgactggcg			
BD131	cagtcgattggctgacctgaagcgctggccgcatc	*Bg*739	1,015	
BD132	gcgcttcaccagcgccag			
BD162	ggttggtgatgatccaggtg	*Bg*739	Table S5	colony PCR
BD163	cctgttcctctgcttcaagg			
BD165	aggaactgctgatcgtccac	*Bg*739	945	cloning of pBD47
BD166	agcctaagcttacacgaaggacatcacgaaacc			
BD167	tgatgtccttcgtgtaagcttaggctgctgccac	pET28a	1,188	
BD168	taggccatgtagctcagaagaactcgtcaagaagg			
BD169	cgagttcttctgagctacatggcctacctgacg	*Bg*739	927	
BD170	agccgtggtagatcatctgg			
BD171	gcgtgttcgagttcttcgac	*Bg*739	Table S5	colony PCR
BD172	gcagaacgcgtcgagatag			
BD173	ctgggcttccatctctatgc	*Bg*739	829	cloning of pBD48
BD174	gcagcctaagcttacaccggcaggaagtggtattc			
BD175	ccacttcctgccggtgtaagcttaggctgctgc	pET28a	1,192	
BD176	gccgtggtagatcattcagaagaactcgtcaagaag			
BD177	gacgagttcttctgaatgatctaccacggctggac	*Bg*739	911	
BD178	cgcaagaaaggcatcgag			
BD179	ctttatcgcgagacccatcc	*Bg*739	Table S5	colony PCR
BD180	atccggacaggaatcttgc			

Table S3. Primers used in this study. *Bg*739, genomic DNA from *B. gladioli* HKI0739 (continued)

Primer	Sequence 3' → 5'	Template	Product size [bp]	Purpose
BD181 BD182	cctgttcctctgcttcaagg gcagcctaagcttaccgtacatcagcgcgaactc	*Bg*739	917	cloning of pBD49
BD183 BD184	tcgcgctgatgtacggtaagcttaggctgctgc ggtttcgttgagcactcagaagaactcgtcaagaag	pET28a	1,192	
BD185 BD186	gacgagttcttctgagtgctcaacgaaacctaccc ggtggtggtcgtagaaatcc	*Bg*739	833	
BD187 BD188	gtacctgcattcgctggtg catgtccgactggatcagc	*Bg*739	Table S5	colony PCR
BD189 BD190	gctgttcacctcgaacttc gcagcctaagcttacacaccagcaggttcttgag	*Bg*739	1,027	cloning of pBD50
BD191 BD192	agaacctgctggtgtgtaagcttaggctgctgc cgttgagcacgtagatcagaagaactcgtcaagaag	pET28a	1,192	
BD193 BD194	gacgagttcttctgatctacgtgctcaacgacc gacttcatccacagcacc	*Bg*739	921	
BD195 BD196	catccatggcagtttcctg cgcacgtgatggttcttg	*Bg*739	Table S5	colony PCR
BD197 BD198	ggcctattccttcgtgctc gcagcctaagcttaccgcacgtgatggttcttg	*Bg*739	905	cloning of pBD51
BD199 BD200	gaaccatcacgtgcggtaagcttaggctgctgc agttgcagctcgacctcagaagaactcgtcaagaag	pET28a	1,192	
BD201 BD202	gacgagttcttctgaggtcgagctgcaactgatg ccacgatcaggatggtcttc	*Bg*739	889	
BD203 BD204	tctacgtgctcaacgacctg gcgccgtagagatagttcg	*Bg*739	Table S5	colony PCR
BD205 BD206	acgagttcttcgcgctgtc gcagcctaagcttacccacgatcaggatggtcttc	*Bg*739	818	cloning of pBD52
BD207 BD208	ccatcctgatcgtgggtaagcttaggctgctgc ggcgtcgaattggagtcagaagaactcgtcaagaag	pET28a	1,192	
BD209 BD210	gacgagttcttctgactccaattcgacgccaac gagctgatgtgcaccgtatg	*Bg*739	994	
BD211 BD212	caggtgctgtggatgaagtc aaccaggcatacacggtcag	*Bg*739	Table S5	colony PCR
BD213 BD214	ctccaattcgacgccaac gcagcctaagcttacgagctgatgtgcaccgtatg	*Bg*739	919	cloning of pBD53
BD215 BD216	ggtgcacatcagctcgtaagcttaggctgctgc gtgtagaaatcgtcgtcagaagaactcgtcaagaag	pET28a	1,192	
BD217 BD218	gacgagttcttctgacgacgatttctacacccagac gcggtcccggtagaaataac	*Bg*739	884	
BD219 BD220	accaccgagacatgaagacc tccaggtaaccgagcttctc	*Bg*739	Table S5	colony PCR
BD332 BD333	tcgactacatcgacctgatcc acaagggcaatacgctgaac	*Bg*739	2,350	cloning of pBD70
BD334 BD335	tgcgcacgtggtaagcttaggctgctgcc agaccacgtgtcagaagaactcgtcaag	pET28a	1,182	
BD336 BD337	catacggtgcacatcagctc cgaaggccagatctcctatg	*Bg*739	Table S5	colony PCR
TIISS_A_rv TIISSD _B_fw Apra_seq_fwd Apra_seq_rv	agtgacaacgtcgagcacag cgttggctacccgtgatatt ggagctgtggaccagcagc ctcgagaatgaccactgc	*Bg*739	Table S5	colony PCR

Table S4. Plasmids constructed in this study.

Plasmid name	Size [bp]	Resistance	Purpose
pBD41	5,894	Kanamycin, ampicillin	Knockout of *spgZ*; *Ara*C gene
pBD42	6,312	Apramycin, kanamycin	Knockout of *spgA*; *Isn*A gene
pBD43	5,819	Apramycin, ampicillin	Knockout of *spgD*; *Isn*A-*IsnB* gene
pBD44	6,364	Apramycin, kanamycin	Knockout of *spgE*; glycosyltransferase gene
pBD45	6,349	Apramycin, kanamycin	Knockout of *spgG*; methyltransferase gene
pBD47	6,054	Kanamycin, ampicillin	Knockout of *spgB*; methyltransferase gene
pBD48	5,846	Kanamycin, ampicillin	Knockout of *spgC*; putative phosphatase gene
pBD49	5,856	Kanamycin, ampicillin	Knockout of *spgF*; methyltransferase gene
pBD50	6,054	Kanamycin, ampicillin	Knockout of *spgH*; transferase gene
pBD51	5,900	Kanamycin, ampicillin	Knockout of *spgI*; oxidase gene
pBD52	5,858	Kanamycin, ampicillin	Knockout of *spgJ*; oxidase gene
pBD53	5,924	Kanamycin, ampicillin	Knockout of *spgK*; oxidase gene
pBD70	6,492	Kanamycin, ampicillin	Knockout of *spgL*; sugar mutase gene

Table S5. Primers used for colony PCR and expected product of sizes and number of the corresponding bands shown in Figure S5 and S6.

Strain name	PCRs					
	PCR-product front arm (F)		PCR-product back arm (B)		PCR-product wild type (C)	
	Primer	Size [bp]; (Band)	Primer	Size [bp] (Band)	Primer	Size [bp] (Band)
*B. gladioli*_pBD41 (Δ*spgZ*; *Ara*C homologue)	BD154 TIISS_A_rv	1,824 (1)	BD155 TIISSD _B_fw	1,239 (2)	BD156 BD155	1,777 (3, 4)
*B. gladioli*_pBD42 (Δ*spgA*; *Isn*A-homologue)	BD156 Apra_seq_fw	1,421 (5)	BD157 Apra_seq_rv	1,546 (6)	BD155 BD156	1,777 (3, 7)
B. gladioli_pBD43 (Δ*spgD*; *Isn*A-*Isn*B homologue)	BD158 Apra_seq_fw	1,410 (8)	BD159 Apra_seq_rv	1,508 (9)	BD159 BD161	1,732 (10, 11)
*B. gladioli*_pBD44 (Δ*spgE*; glycosyltransferase gene)	BD160 Apra_seq_rv	1,679 (12)	BD161 Apra_seq_fwd	1,582 (13)	BD159 BD161	1,732 (11, 14)
*B. gladioli*_pBD45 (Δ*spgG*; methyltransferase gene**)**	BD162 Apra_seq_rv	1,463 (14)	BD163 Apra_seq_fwd	1,542 (16)	BD164 BD162	1,717 (15, 18)

Strain name	PCR product mutant strain (mut)		PCR-product wild type (wt)	
	Primer	Size [bp] (Band)	Primer	Size [bp] (Band)
*B. gladioli*_pBD47 (Δ*spgB*; transporter gene)	BD249 BD250	3,092 (20)	BD249 BD250	2,190 (19)
*B. gladioli*_pBD48 (Δ*spgC*; putative phosphatase)	BD 179 BD180	3,029 (21)	BD 179 BD180	2,007 (22)
*B. gladioli*_pBD49 *(ΔspgF;* hypothetical gene*)*	BD351 BD352	3,357 (23)	BD351 BD352	2,400 (24)
*B. gladioli*_pBD50 (Δ*spgH*; transferase gene**)**	BD195 BD196	3,485 (25)	BD195 BD196	2,419 (26)
*B. gladioli*_pBD51 *(ΔspgI;* oxidase gene*)*	BD348 BD204	3,690 (27)	BD348 BD204	2,500 (28)
*B. gladioli*_pBD52 *(ΔspgJ;* reductase gene*)*	BD211 BD212	3,719 (29)	BD211 BD212	2,700 (30)
*B. gladioli*_pBD53 *(ΔspgK;* dehydratase*)*	BD219 BD220	3,440 (31)	BD219 BD220	2,299 (32)
*B. gladioli*_pBD70 *(ΔspgL;* sugar mutase*)*	BD324 BD325	3,995 (33)	BD324 BD325	3,101 (34)

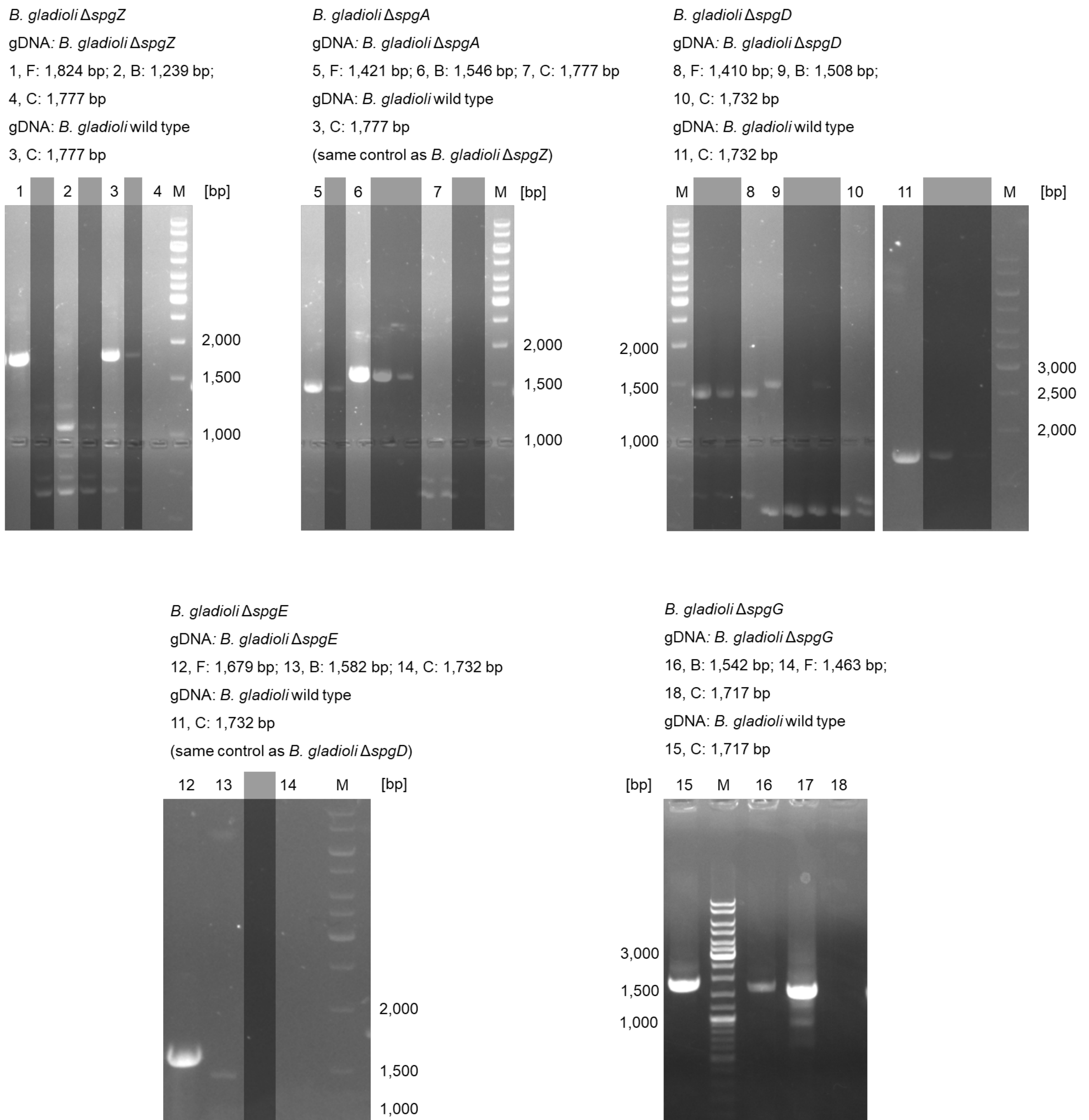
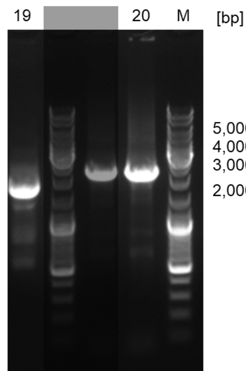
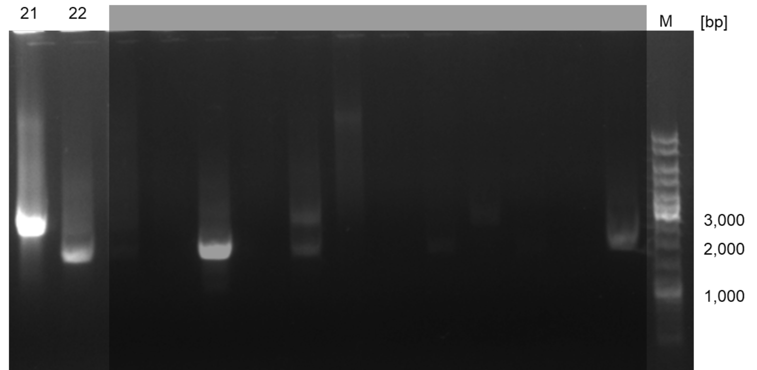


Figure S5. PCR-based verification of genetic manipulations of *B. gladioli* HKI0739. Sizes of expected PCR-products F, B and C are listed in Table S5. Irrelevant bands are covered with grey boxes.

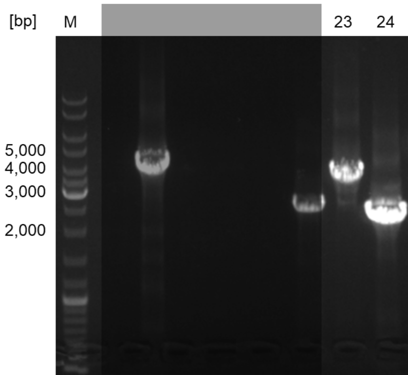
B. gladioli Δ*spgB*
gDNA: *B. gladioli* Δ*spgB*
20: 3,092 bp
gDNA: *B. gladioli* wild type
19: 2,190 bp



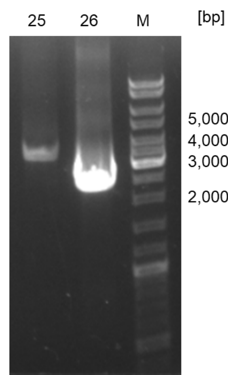
B. gladioli Δ*spgC*
gDNA: *B. gladioli* Δ*spgC*
21: 3,029 bp
gDNA: *B. gladioli* wild type
22: 2,007 bp



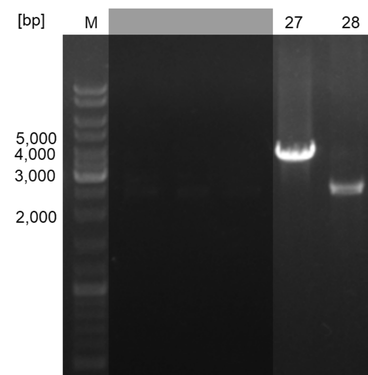
B. gladioli Δ*spgF*
gDNA: *B. gladioli* Δ*spgF*
23: 3,357 bp
gDNA: *B. gladioli* wild type
24: 2,400 bp



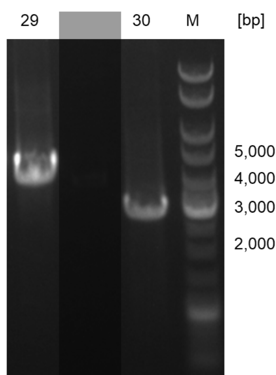
B. gladioli Δ*spgH*
gDNA: *B. gladioli* Δ*spgH*
25: 3,485 bp
gDNA: *B. gladioli* wild type
26: 2,419 bp



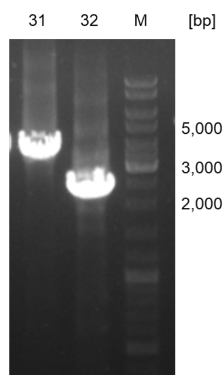
B. gladioli Δ*spgI*
gDNA: *B. gladioli* Δ*spgI*
27: 3,690 bp
gDNA: *B. gladioli* wild type
28: 2,500 bp



B. gladioli Δ*spgJ*
gDNA: *B. gladioli* Δ*spgJ*
29: 3,719 bp
gDNA: *B. gladioli* wild type
30: 2,700 bp



B. gladioli Δ*spgK*
gDNA: *B. gladioli* Δ*spgK*
31: 3,440 bp
gDNA: *B. gladioli* wild type
32: 2,299 bp



B. gladioli Δ*spgL*
gDNA: *B. gladioli* Δ*spgL*
33: 3,995 bp
gDNA: *B. gladioli* wild type
34: 3,101 bp

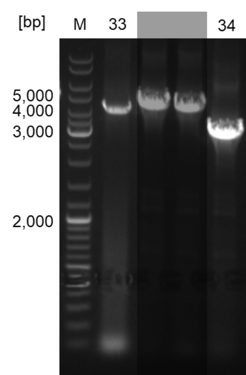


Figure S6. PCR-based verification of genetic manipulations of *B. gladioli* HKI0739. Sizes of expected PCR-products are listed in Table S5. Irrelevant bands are covered with grey boxes. (continued)

Biological assays

Antifungal assays

The antifungal activity of **13** was tested in an agar diffusion assay. 50 µL of a solution of **13** (1 mg mL^{-1} in DMSO) were filled in holes (9 mm diameter) of a PDA plate, inoculated with a spore suspension of either *Aspergillus fumigatus*, *Penicillium notatum* or *Purpureocillium lilacinum,* respectively. After incubation at 30 °C for 24 h, the inhibition zone was measured.[8] Ketozonazol (1.5 mg mL^{-1}, in DMSO) was used as a positive control for tests against fungi. No inhibition zones were observed (Figure S7).

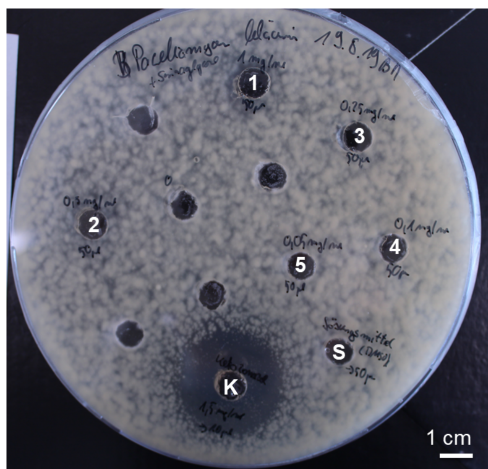


Figure S7. Activity of **13** against *Purpureocillium lilacinum*, a natural fungal antagonist of *Lagria villosa*. Wells 1–5 correspond to **13** at a concentration of 1, 0.5, 0.25, 0.1, 0.05, mg mL^{-1} in dimethyl sulfoxide (DMSO). Well S corresponds to a negative control (solvent, DMSO) and K to the positive control (Ketoconazol, 1.5 mg mL^{-1}).

Beetle egg infection experiments

A total of 180 *Lagria villosa* eggs from three clutches were surface sterilized to remove the microbial community as described previously.[1] Briefly, eggs were submerged in 70% ethanol for 5 min, followed by 30 s in 12% NaClO and a final rinse with sterile water. Each clutch was divided into three groups of equal size (20x) and randomly assigned to the following treatments. The first group remained symbiont-free and 2.5 µL of sterile PBS was applied to each egg. The second group was reinfected with 2.5 µL of a *B. gladioli* HKI0739–WT cell suspension (2×10^6 cells µL^{-1}) and the third with an equivalent amount of the *ΔspgD* mutant strain. 96-well plates containing moist vermiculite and filter discs were prepared by adding 4 µL per well of a *P. lilacinum* spore suspension (10 spores µL^{-1}) in sterile water. Individual eggs were randomly arranged in each well, incubated at 25 °C and monitored blindly during five days to assess fungal growth directly on the egg surface. A Cox Mixed Effects Models with a random intercept per clutch was used to analyse the effect of treatment on fungal growth using the "coxme" package in R (v 3.6.2) (Figure S8).

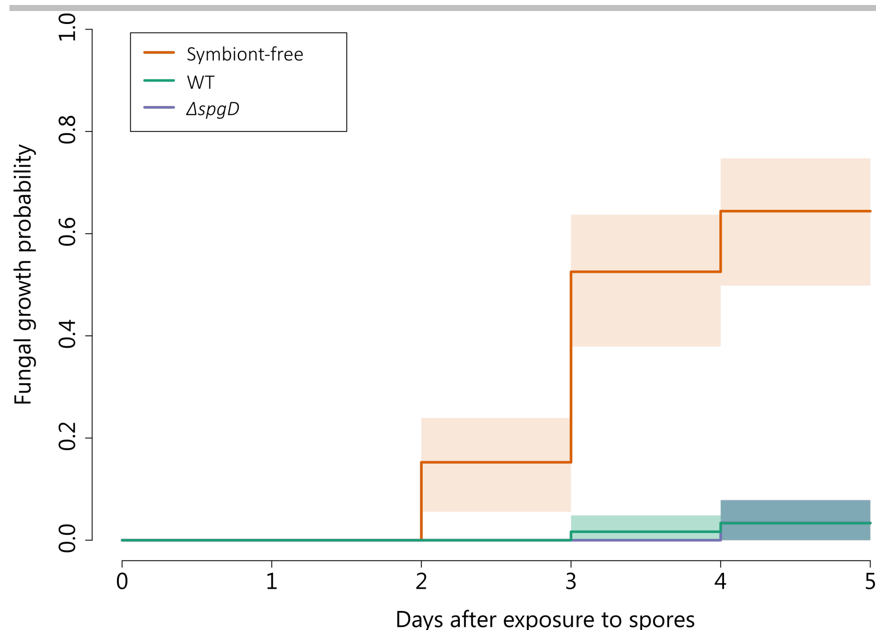


Figure S8. Growth probability of *Purpureocillium lilacinum* on *Lagria villosa* eggs treated with the *B. gladioli* symbiont (WT) , the corresponding *ΔspgD* mutant strain or symbiont-free controls (N = 60 per treatment). A Cox Mixed-Effects Model revealed significant differences between symbiont free and symbiont-infected treatments ($p < 0.001$), yet no difference between the effects of WT and mutant strains ($p > 0.05$). Shadings correspond to 95% confidence intervals.

Isotope labeling experiments

1 mL of a *B. gladioli* HKI 0739 overnight culture was added to a 50 mL PDB culture. After 4, 8 and 12 h of cultivation time, 4 mg of C_3-^{13}C-labeled tyrosine, C_1-^{13}C- labeled tyrosine, ^{15}N-tyrosine (99% ^{13}C, CAMPRO SCIENTIFIC, Germany) in 250 µL ethanol or 3 mg ^{13}C-glucose (99% ^{13}C, CAMPRO SCIENTIFIC, Germany) were added, respectively. LC/MS profiles of the cultures showed the expected mass shifts (Figure S9 and Figure S10).

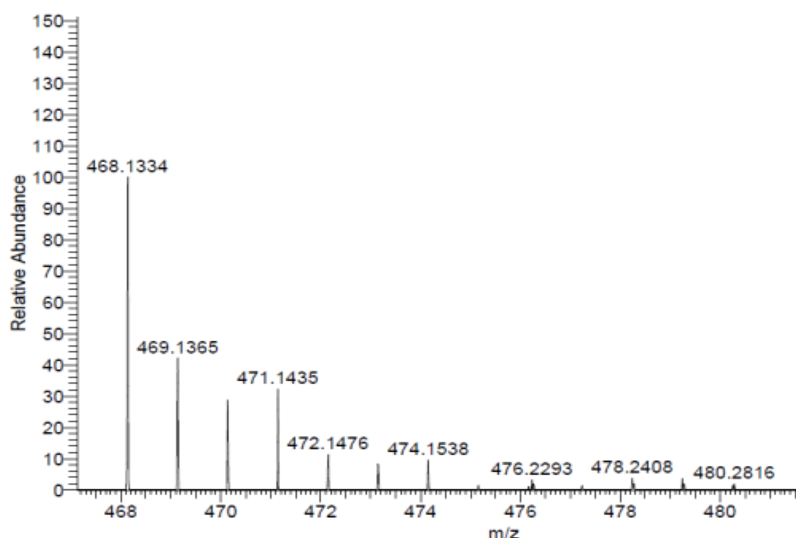


Figure S9. Isotopic pattern of sinapigladioside (**9**), *m/z* 468.1334 $[M–H]^-$ measured from an extract of a *B. gladioli* HKI 0739 culture, grown in a PDB to which ^{13}C-glucose was added in defined intervals.

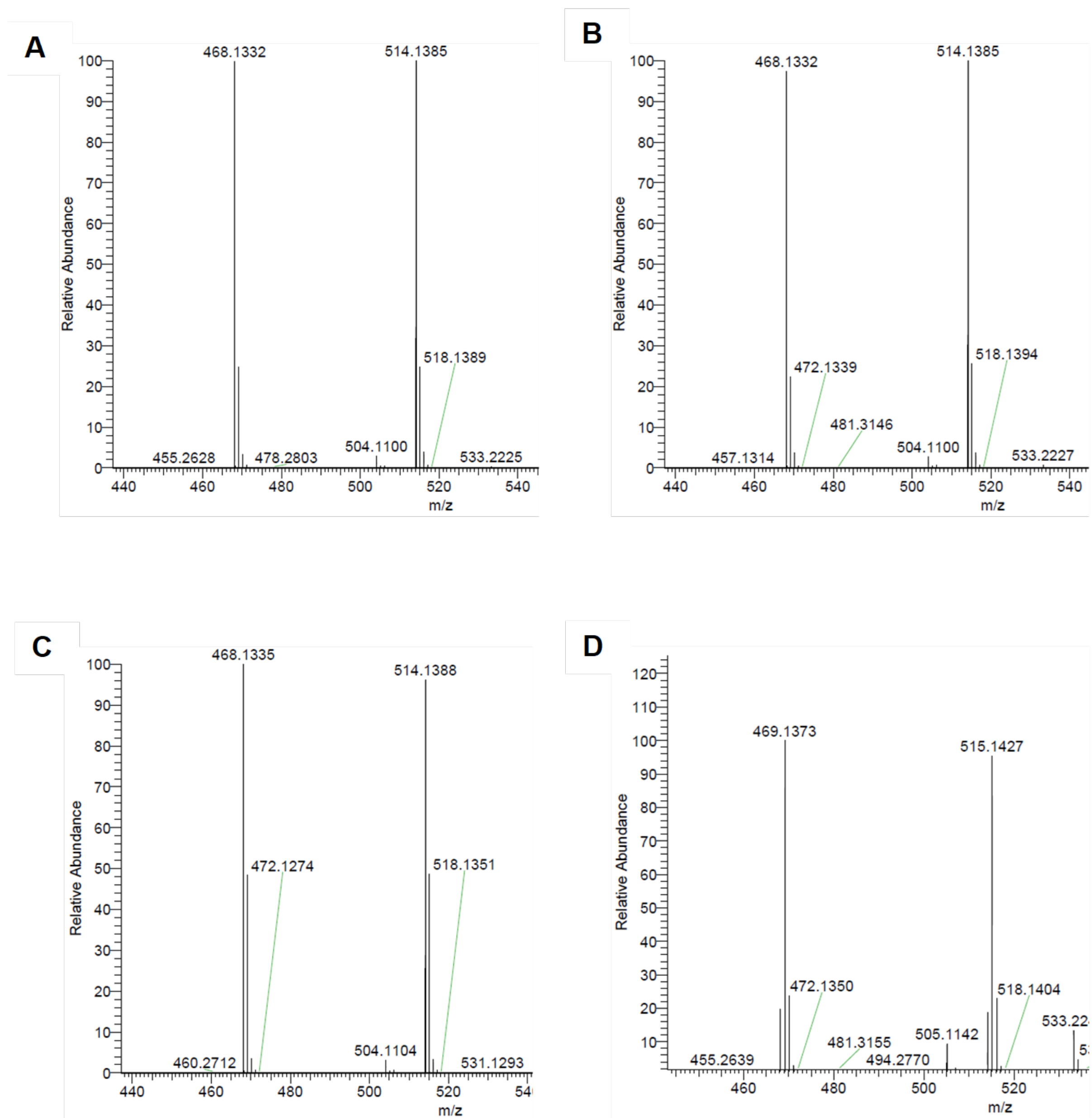


Figure S10. Isotopic pattern of sinapigladioside (**9**), *m/z* 468.1332 $[M–H]^-$ measured from an extract of a *B. gladioli* HKI 0739 culture, grown in PDB to which (A) ethanol (negative control), (B) $^{13}C_1$-tyrosine, (C) ^{15}N-tyrosine, and (D) $^{13}C_3$-tyrosine was added in defined intervals. *m/z* 514.14 corresponds to $[M+COO]^-$.

1D and 2D NMR spectra and LC-MS profiles

Structure elucidation of the biosynthetic intermediate 13

The structure of **13** was elucidated by 1D and 2D NMR analyses (Figure S11 and Table S6).

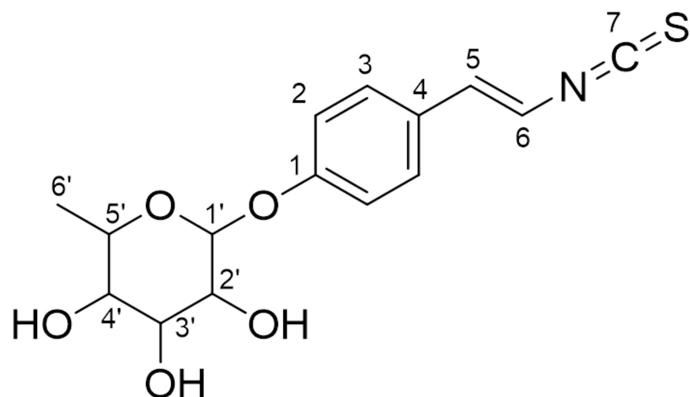


Figure S11. Structure of the sinapigladioside intermediate (**13**).

Table S6. ^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR shifts of **13**.

Position	δ_C [ppm]	δ_H [ppm]; Signal (*J* [Hz])
1	156.6	-
2	116.7	7.02; 2 H d*
3	128.0	7.41; 2 H d (*8.7*)
4	127.2	-
5	131.8	6.86; 1 H d (*13.8*)
6	114.0	7.01; 1 H d*
7	131.4	-
1’	98.1	5.40; 1 H d (*1.5*)
2’	70.1	3.80; 1 H m
3’	70.4	3.62; 1 H m
4’	71.7	3.26; 1 H m
5’	69.6	3.42; 1 H m
6’	17.9	1.08; 3 H d (*6.3*)
2’ OH	-	5.04; 1 H d (*4.4*)
3’ OH	-	4.72; 1 H d (*6.0*)
4’ OH	-	4.85; 1 H d (*5.8*)

* Signal overlay

$[\alpha]^{25, D}$ = –90.1 (*c* = 2, 83% MeCN)

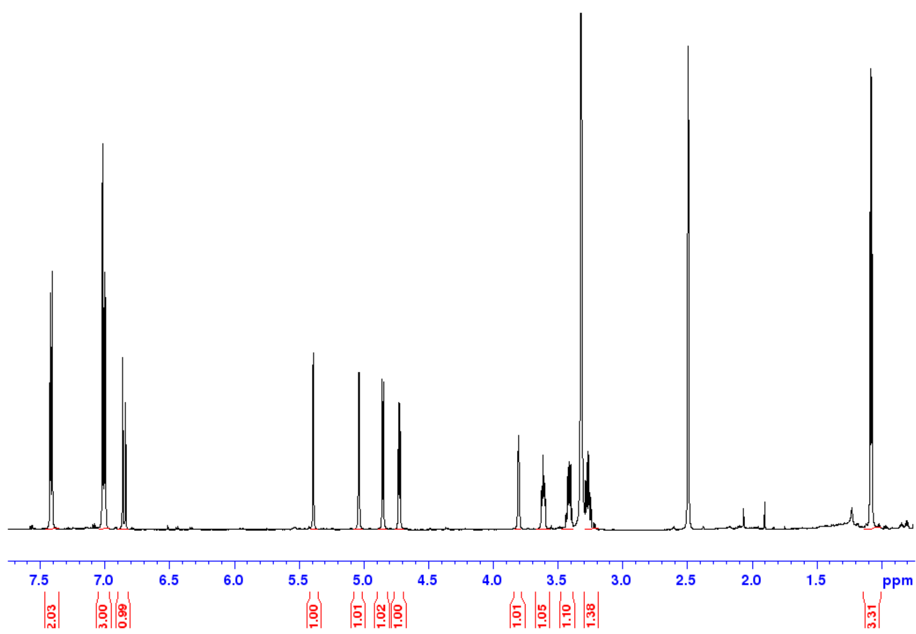


Figure S12. ^{1}H-NMR-spectrum of **13**, recorded at 600 MHz in DMSO-d_6.

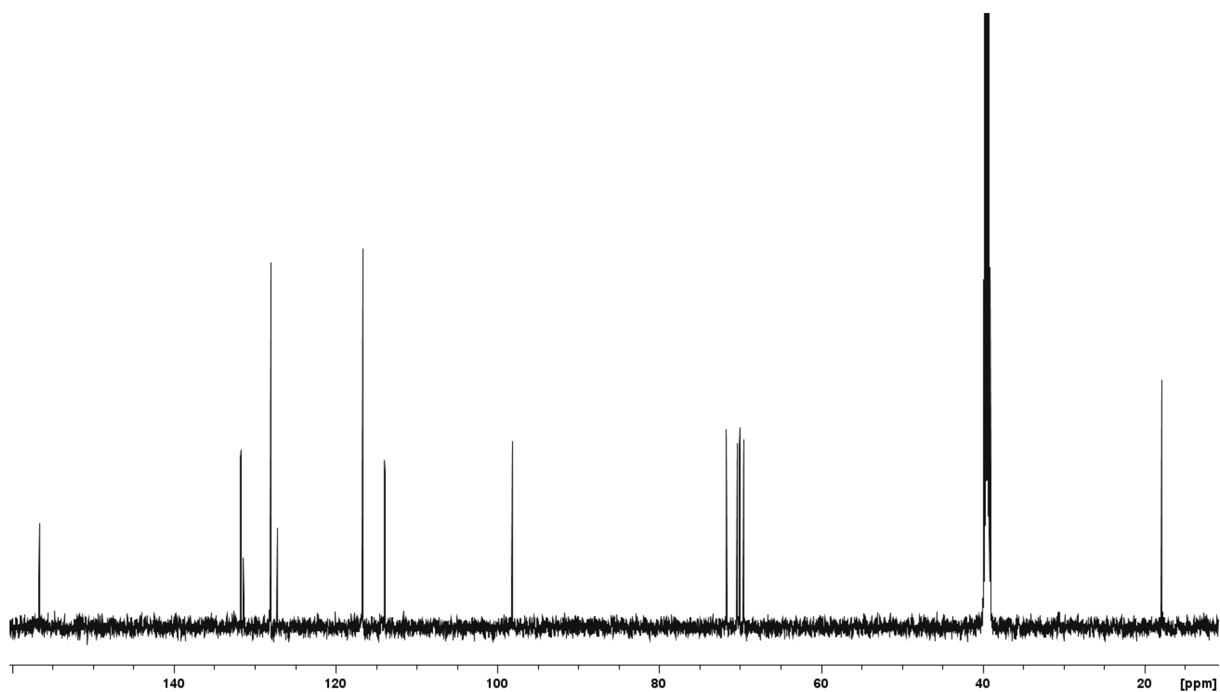


Figure S13. ^{13}C-NMR-spectrum of **13**, recorded at 150 MHz in DMSO-d_6.

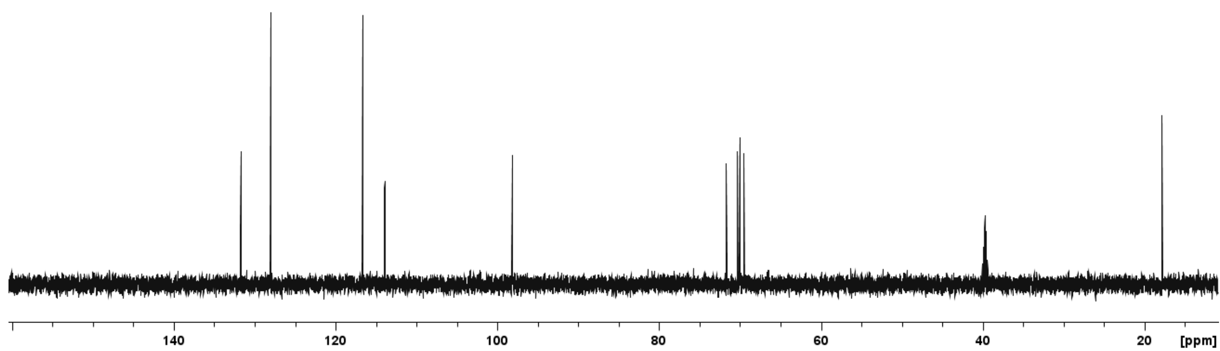


Figure S14. DEPT-135-NMR-spectrum of **13**, recorded at 600 MHz in DMSO-d6.

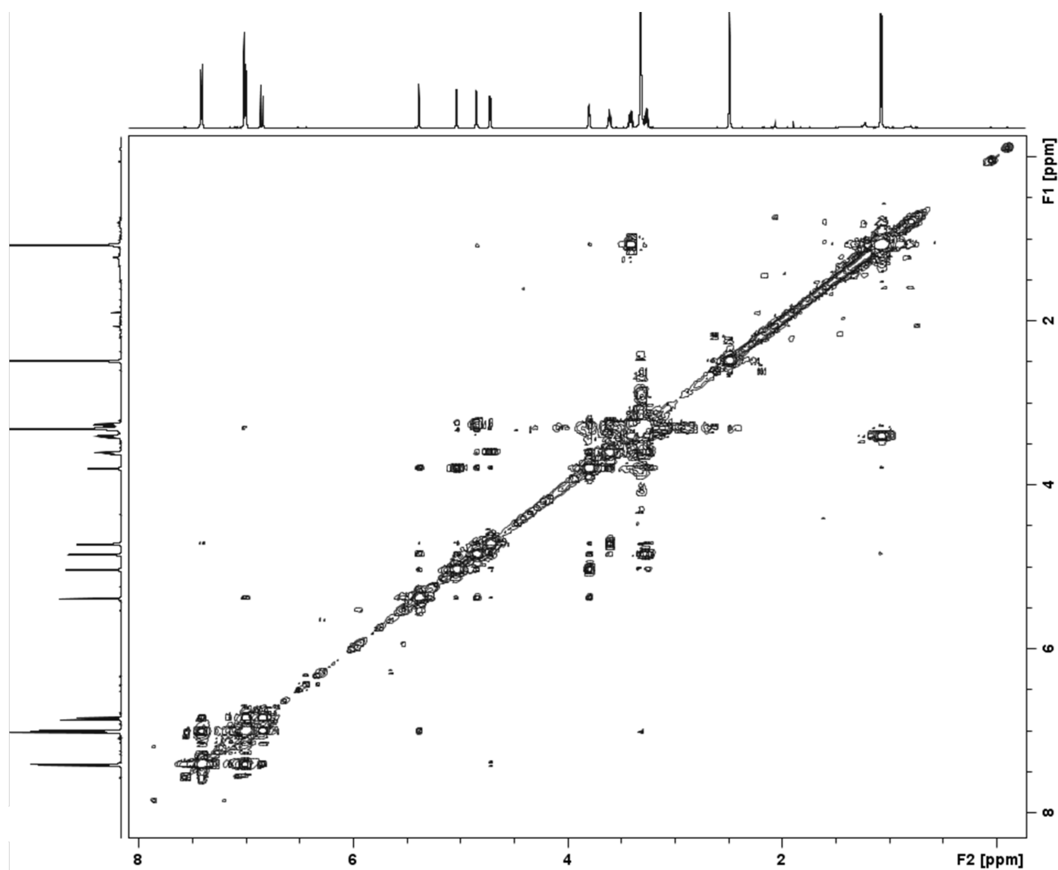


Figure S15. ^{1}H-^{1}H-COSY-NMR-spectrum of **13**, recorded at 600 MHz in DMSO-d6.

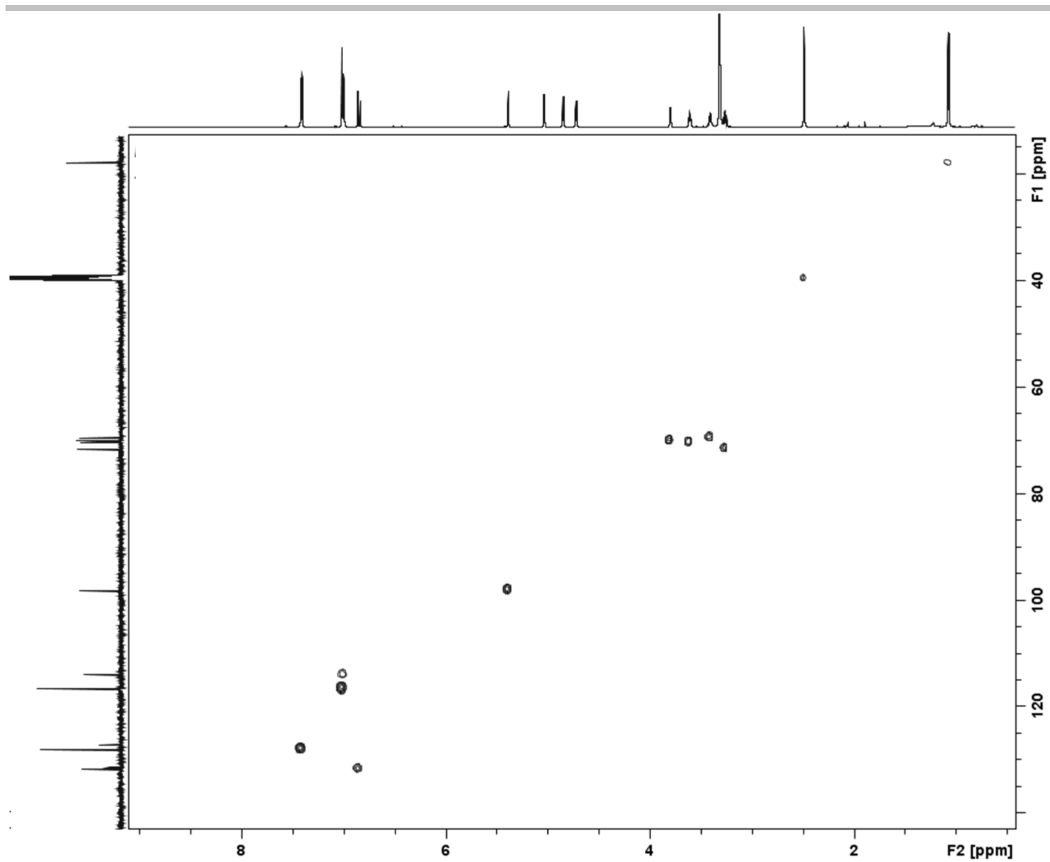


Figure S16. ^{1}H-^{13}C-HSQC-NMR-spectrum of **13**, recorded at 600 MHz in DMSO-d$_6$.

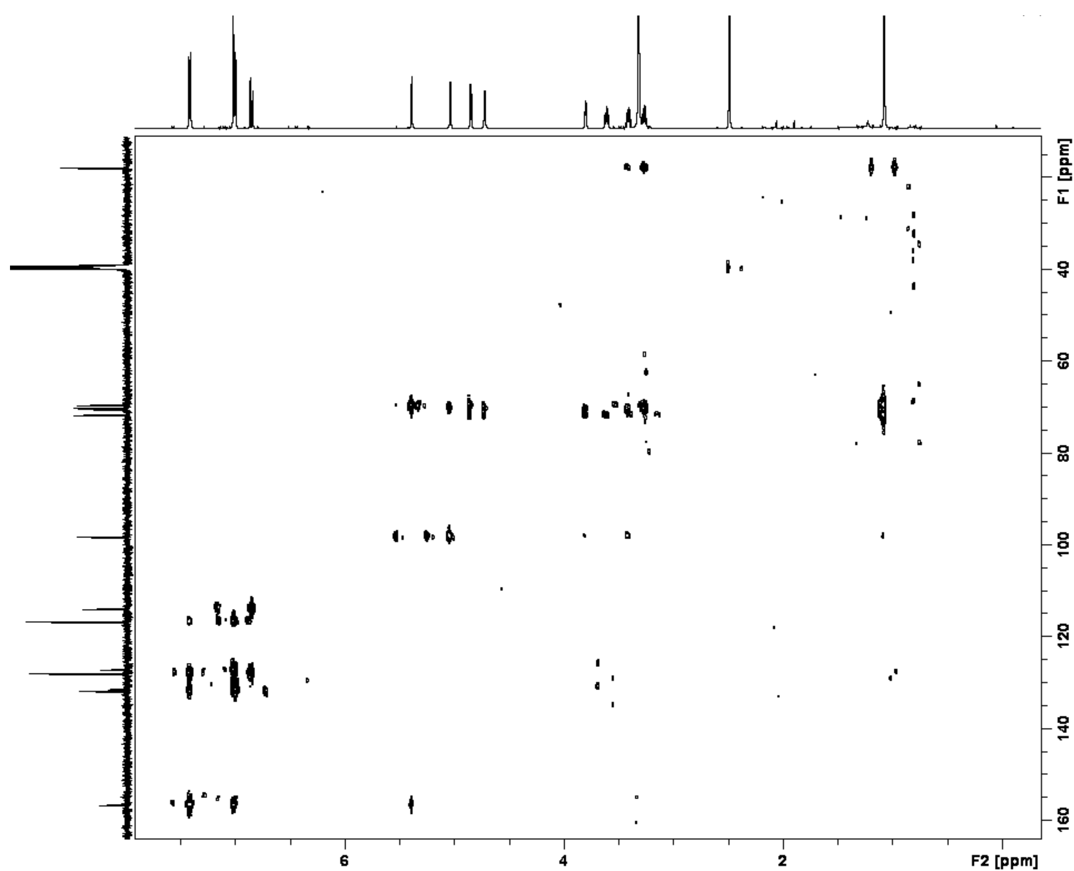


Figure S17. ^{1}H-^{13}C-HMBC-NMR-spectrum of 13, recorded at 600 MHz in DMSO-d$_6$.

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Author Contributions

B.D. performed biological experiments and bioinformatic analysis. B.D. and S.S. constructed gene knockout mutants. S.P.N. isolated and purified compound **13** and performed chemical characterization as well as isotope-labeling studies. L.F. and M.K. performed additional bioactivity assays. All authors contributed to manuscript preparation. C.H. wrote the final version.