Supporting Information

Identification and Characterization of the Chaetoviridin and Chaetomugilin Gene Cluster in *Chaetomium globosum* Reveal Dual Functions of an Iterative Highly-Reducing Polyketide Synthase

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Experimental Procedures

Strains

Chaetomium globosum CBS 148.51 was obtained from The American Type Culture Collection. *Chaetomium globosum ΔCgligD*[1] was used for all gene inactivation experiments. *Saccharomyces cerevisiae* BY4741 was used for constructing the *caz*E inactivation cassette. *Escherichia coli* BL21(DE3) was used for expressing the acyltransferase CazE. *Saccharomyces cerevisiae* BJ5464-NpgA[2] (*MATα ura3-52 trp1 leu2-Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*), which contains a chromosomal copy of the phosphopantetheinyl transferase NpgA,[3] was used for expressing the HR-PKS CazF.

Culture conditions

Chaetomium globosum CBS 148.51 was cultured in 1 L of PD medium (24 g Potato Dextrose from Fluka Analytical and Milli-Q filtered deionized water) in 150 x 15 mm petri dishes. Each plate contained 75 mL of media and were incubated at 28^{o}C for 3–7 days. Inoculation of each plate was carried-out with 1% of a 1 x 10^8 spore suspension. For the [1,2-$^{13}C_2$]acetate feeding study, 100 mg of labeled precursor was added to 1 L of PD medium at the time of inoculation. To purify cazisochromene **8** for structure elucidation and in vitro assays, the Δ*lig*D *C. globosum* strain was cultured in 1 L of MYG medium (10 g/L malt extract, 4 g/L yeast extract, 4 g/L glucose). Cultivation was carried out at 30 ^{o}C for four days at 250 rpm.

Chemicals and spectral analysis

Labeled [1,2-$^{13}C_2$]acetate was purchased from Cambridge Isotope Laboratories, Inc. The 4-Hydroxy-5,6-dimethylpyran-2-one standard for in vitro assays was prepared as described previously.[4] All solvents and other chemicals used were of analytical grade. ^{1}H NMR spectra were obtained on Bruker 400-MHz and 500-MHz spectrometers a 500 MHz Bruker AV500 spectrometer and a JEOL JNM-ECA 500 MHz spectrometer (125 MHz for ^{13}C). ^{13}C NMR spectrum for [1,2-$^{13}C_2$]acetate-enriched chaetomugilin A was obtained on a 500 MHz Bruker AV500 spectrometer with a 5mm dual cryoprobe.

HPLC analysis and purification of chaetoviridins and chaetomugilins

All petri dishes containing PD media were pooled together and the mycelium and media were extracted with ethyl acetate, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. From the 1L culture, the crude extract was fractionated using reverse phase C18 flash column chromatography (Fisher Scientific, PrepSep C18 1 g/6 ml) with 20% MeCN:H_2O, 40% MeCN:H_2O, 60% MeCN:H_2O, 80% MeCN:H_2O, and 100% MeCN as the mobile phase. The fractions were analyzed in positive and negative mode on a Shimadzu 2010 EV Liquid Chromatography Mass Spectrometer (gas flow set to 4.2 mL/min, drying temperature set to 200 °C, and capillary voltage set to 2000 V). A Luna C18(2) 100 x 2 mm column was used with a flow rate of 0.1 mL/min and a linear solvent gradient of 5–95% MeCN:H_2O containing 0.1% formic acid over a period of 30 min followed by 15 minutes of 95% MeCH:H_2O.

Isolation of cazisochromene (8)

The MYG culture was centrifuged and the supernatant was extracted with 2 x 1 L volumes of ethyl acetate. The ethyl acetate extracts were combined and concentrated *in vacuo,* which yielded an oily residue. The residue was fractionated by silica gel flash column chromatography using 99% $CHCl_3$:MeOH. Fractions containing the target product were collected and further purified by reverse phase HPLC (Nacalai Tesque Inc.) using a COSMOSIL 5 C18 MS-II 5µm 20 x 250 mm column. Compound **8** was isolated at 1 mg/L and its chemical structure was elucidated from the spectroscopic data given in SI Table 4.

Isolation of chaetomugilin A (4) and chaetomugililn M (7)

The PD cultures were extracted after seven days of incubation. Compound **4** and **7** eluted in the 40% MeCN:H_2O fraction and were purified by HPLC (Beckman Coulter) using a Luna C18 column (250 x 10 mm; 5µm particle size) and an isocratic condition of 45% MeCN:H_2O containing 0.1% TFA with a flow rate of 2.0 mL/min. The t_R of compound **4** was 13 min and the t_R of compound **7** was 23 min. The coupling constants for [1,2-$^{13}C_2$]-enriched **4** are shown in SI Table 2.

Isolation of chaetoviridin A (3), chaetoviridin B (5) and chaetoviridin C (6)

The PD cultures were extracted after three days of incubation. The azaphilone metabolites **3**, **5**, and **6** eluted in the 60% MeCN:H_2O fraction described above and were purified by HPLC (Beckman Coulter) using a Luna C18 column (250 x 10 mm; 5µm particle size) and an isocratic condition of 50% MeCN:H_2O containing 0.1% TFA with a flow rate of 2.0 mL/min. The t_R of **3**, **5**, and **6** were 35 minutes, 28 minutes and 32 minutes, respectively.

General molecular biology experiments

PCR reactions were carried out using Platinum Pfx DNA polymerase (Invitrogen), Phusion DNA polymerase (New England Biolabs), and PrimeSTAR GXL DNA polymerase (Takara). Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. pCR®-blunt (Invitrogen) was used to construct recombinant DNA products and DNA sequencing was performed by Retrogen, California, USA and by Macrogen Japan Corporation. DNA manipulation using standard techniques[5] were performed in *E. coli* TOP10 (Invitrogen) and *E. coli* XL1-Blue (Stratagene).

Construction of gene inactivation cassettes

Disruption cassettes containing the hygromycin phosphotransferase *hph* gene, which confers resistance to hygromycin, and the *tub* promoter were designed by fusion PCR[6] for *caz*F, and *caz*M. The *hph* resistance marker was amplified from plasmid pKW3202[7] using primer pair cazF_hpfF and cazF_hpfR for inactivation of *caz*F and primer pair cazM_hpfF and cazM_hpfR for inactivation of *caz*M. The knockout cassettes were constructed as previously described[6] except 2 kb of homologous DNA located upstream and downstream of the targeted gene were attached to the resistance marker for the *caz*F inactivation, whereas 1 kb was attached for the *caz*M cassette. For construction of the *caz*F cassette, primer pairs CazF_ko_P1 and CazF_ko_P2 were used for the upstream region, while CazF_ko_P3 and CazF_ko_P4 were used for the downstream region. For inactivation of *caz*M, primer pairs CazM_ko_P1 with CazM_ko_P2 and CazM_ko_P3 with CazM_ko_P4 were used for constructing the upstream and downstream regions, respectively.

The disruption cassette for inactivation of *caz*E was also designed to contain the *hph* resistance marker and amplified from the pKW3202 cassette using primer pairs CazE_hphF and CazE_hphR. The homologous upstream and downstream regions (1 kb) were amplified from genomic DNA (gDNA) using primer pairs CazE_ko_P1 with CazE_ko_P2 and CazE_ko_P3 with CazE_ko_P4, respectively. For in vivo homologous recombination in yeast, the P1–P2 region, P3–P4 region and *hph* resistance cassette (50 ng to 150 ng each) were mixed with 40 ng of the linearized delivery vector pRS426[7] in a total volume of 45 µL. pRS426 was linearized by digesting with *Kpn* I and *Sac* I at 37 ^{o}C for eight hours. The mixture was transformed into *S. cerevisiae* BY4741 and the four DNA fragments were joined *in situ* by the endogenous homologous recombination activity of *S. cerevisiae* through the 15-bp homologous sequences present in the DNA fragments. The desired transformants were selected on a uracil-deficient plate using the presence of the selection marker *URA*$_3$. The resulting plasmid pKW19053 containing the *cazE-hph* gene inactivation cassette was recovered from the yeast transformant and transferred to *E. coli*. pKW19053 was amplified in *E. coli* for subsequent characterization by

restriction enzyme digestion and DNA sequencing to confirm its identity. The disruption cassette was amplified from pKW19053 using primer pair cazE_ko_P1 and cazE_ko_P4.

Transformation of disruption cassettes into C. globosum ΔligD

Linearized inactivation cassettes were transformed into *C. globosum* Δ*lig*D as described previously.[7] Transformants were grown on MYG agar supplemented with 200 µg/mL hygromycin B. To confirm correct integration of the disruption cassettes into the genome, gDNA was extracted from the transformants and used as template for PCR using primer pairs CazE_mt_ver_P0 with CazE_mt_ver_P5, CazE_mt_ver_P6 with CazE_mt_ver_P7, CazF_mt ver_P0 with CazF_mt_ver_P5, CazF_mt_ver_P6 with CazF_mt_ver_P7, CazM_mt_ver_P0 with CazM_mt_ver_P5, and CazM_mt_ver_P6 with CazM_mt_ver_P7 (Figure S3).

Protein expression and purification

*caz*E did not contain any introns and was amplified from *C. globosum* gDNA using primer pair CazE_F and CazE_R, which contained EcoRI and NotI restriction sites, respectively. The PCR product was digested with EcoRI and NotI and ligated into the pHis8 expression vector,[8] which was previously digested with the same restriction enzymes. The expression plasmid pJW07637 was transformed into *E. coli* BL21 (DE3) electro competent cells. A single colony was used to inoculate 10 mL Luria-Bertani (LB) media supplemented with 35 µg/mL kanamycin and grown overnight with constant shaking at 37 ^{o}C. A 5 mL aliquot of the seed culture was used to inoculate 1 L of Terrific Broth (TB) (24 g yeast extract, 12 g tryptone and 4 mL glycerol) supplemented with 35 mg/L kanamycin. The cells were grown at 37 ^{o}C to an OD600 of 0.4. To induce protein expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the culture was shaken at 16 ^{o}C for 16 hours. The cells were harvested by centrifugation (3,750 rpm at 4 ^{o}C for 20 mins) and the cell pellet was resuspended in 20 mL lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 2 mM DTT, 500 mM NaCl, 5 mM imidazole, pH 7.9). The cells were sonicated on ice in 30 second intervals until homogenous. To remove cellular debris, the homogenous mixture was centrifuged at 17,000 rpm for 20 min at 4 ^{o}C. Ni-NTA agarose resin was added to the supernatant (1 mL) and the solution was incubated at 4 ^{o}C for 4 hours. Soluble CazE was purified by gravity-flow column chromatography using increasing concentrations of imidazole in buffer A (50 mM Tris-HCl, 500 mM NaCl, 20 mM–250 mM imidazole, pH 7.9). Purified CazE was concentrated and buffer exchanged into Buffer B (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, pH 8.0) using an Ultracel 30,000 MWCO centrifugal filter (Amicon Inc.) and stored in 10% glycerol. The protein concentration was calculated to be 52 mg/L by Bradford assay using BSA as a standard.

The original DNA sequence of *caz*F contained a large unsequenced gap. Primer pair Nseq_07638fwd and Nseq_07638rev was used to amplify this region, which was subcloned into pCR®-blunt and sequenced (Figure S9). To reconstitute an intron-free *caz*F, four overlapping pieces were constructed (Figure S10). Piece two (2,585 bp) and piece four (3,194) did not contain any introns and were amplified from gDNA using primer pairs CazF_P2_F with CazF_P2_R and CazF_P4_F with CazF_P4_R, respectively. Piece one and three were initially annotated to contain introns and were thus amplified from cDNA. RNA was extracted from a five-day old PD culture using the RiboPure Yeast kit (Ambion) following the manufacturer's instructions and gDNA was digested with DNase (2 U/µL) (Invitrogen) at 37 ^{o}C for four hours. Complementary DNA (cDNA) was synthesized from total RNA for piece one and three using SuperScript III First Strand Synthesis System with the reverse primers CazF_P1_R and CazF_P3_R, respectively. The cDNA was used as template for PCR and piece one and three were amplified using primer pairs CazF_P1_F with CazF_P1_R and CazF_P3_F with CazF_P3_R. The four exons were co-transformed with the 2 *µm* expression plasmid, which was previously linearized by digesting with Pml I and Nde I at 37 ^{o}C overnight, in *S. cerevisiae*

BJ5464-NpgA using an *S. c* EasyComp™ Transformation kit (Invitrogen) to yield the expression plasmid pJW07638. A single transformant was used to inoculate 3 mL SD_{ct} media (0.5 g Bacto Technical grade cassamino acids, 2 g Dextrose, and 88 mL Milli-Q water) supplemented with 1 mL adenine (40 mg/20 mL Milli-Q water), 1 mL tryptophan (40 mg/20 mL Milli-Q water) and 10 mL nitrogen base (1.7 g Nitrogen Base without amino acids, 5 g ammonium sulfate and 100 mL Milli-Q water) and grown for 72 hours with constant shaking at 28 ^{o}C. A 1 mL aliquot of the seed culture was used to inoculate 1 L of YPD (10 g yeast extract, 20 g peptone and 950 mL Milli-Q water) supplemented with 1% dextrose and the culture was shaken at 28 ^{o}C for 72 hours. The cells were harvested by centrifugation (3,750 rpm at 4 ^{o}C for 10 mins) and the cell pellet was resuspended in 30 mL lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 10 mM imidazole, pH 8.0). The cells were sonicated on ice in one minute intervals until homogenous. To remove cellular debris, the homogenous mixture was centrifuged at 17,000 rpm for 1 hour at 4 ^{o}C. Ni-NTA agarose resin was added to the supernatant (2 mL) and the solution was incubated at 4 ^{o}C for 12–16 hours. Soluble CazF was purified by gravity-flow column chromatography using increasing concentrations of imidazole in buffer A (50 mM Tris-HCl, 500 mM NaCl, 20 mM–250 mM imidazole, pH 7.9). Purified protein was concentrated and buffer exchanged into Buffer B (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, pH 8.0) using an Ultracel 100,000 MWCO centrifugal filter (Amicon Inc.) and stored in 10% glycerol. The sequence of an intron-free *caz*F was confirmed using primers CazF_seq1–CazF_seq6 and its protein concentration was calculated to be 9.5 mg/L using the Bradford assay with BSA as a standard.

CazF in vitro activity assays

To assess the minimal activity of CazF, 25 μM of protein was incubated at room temperature with 2 mM malonyl-CoA in 100 mM phosphate buffer pH 7.4 in a total volume of 100 μL. After 16 hours, the reaction was base hydrolyzed by adding 20 μL 1M NaOH and incubated at 65 ^{o}C for 10 minutes. The reaction was then extracted two times with 200 μL 99% ethyl acetate:1% acetic acid and the organic layer was dried by speedvac. The extract was then dissolved in 20 μL MeOH and analyzed on a Shimadzu 2010 EV LC-MS with a Phenomenex Luna 5μ 2.0 x 100 mM C18 column using positive and negative mode electrospray ionization with a linear gradient of 5–95% MeCN:H_2O over 30 minutes followed by 95% MeCN for 15 minutes and a flow rate of 0.1 mL/min. When the reaction was only extracted with ethyl acetate and not base hydrolyzed, lower levels of the triketide alpha-pyrone (**13**) was detected by LC-MS. However, when 25 μM of CazE was added to the reaction and extracted by ethyl acetate, comparable amounts of **13** were detected by LC-MS (Figure S12).

The activity of the methyltransferase domain in CazF was analyzed by adding *S*-adenosyl-L-methionine chloride (SAM) to the minimal PKS activity assay. In a 100 μL reaction, 25 μM CazF was incubated with 2 mM SAM and 2 mM malonyl-CoA in 100 mM phosphate buffer pH 7.4. The reaction was incubated at room temperature for 16 hours, base hydrolyzed, extracted with 99% ethyl acetate:1% acetic acid, resuspended in 20 μL MeOH and analyzed by LC-MS as mentioned above.

The reduction activities of the ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains were analyzed by incubating 25 μM CazF with 2 mM NADPH, 2 mM acetoacetyl-SNAC and 100 mM phosphate buffer pH 7.4 in a total volume of 100 μL. The reaction was incubated at room temperature for 12 hours, extracted two times with 200 μL ethyl acetate and the organic layer was dried by speedvac. The extract was resuspended in 20 μL MeOH and analyzed by LC-MS using the same conditions mentioned above (Figure S15).

In vitro biosynthesis of chaetoviridin A (3) and 4'-desmethyl-chaetoviridin A (15) using CazF, CazE and cazisochromene (8)

In a 100 μL reaction, 25 μM CazF was incubated with 2 mM NADPH, 2 mM SAM, 2 mM malonyl-CoA, 25 μM CazE and 2 mM **8** in 100 mM phosphate buffer pH 7.4. The reaction was incubated at room temperature for three hours and extracted two times with 200 μL ethyl acetate. The organic layer was dried by speedvac, resuspended in 20 μL MeOH and analyzed by LC-MS using the same conditions in the CazF in vitro activity assays. The in vitro biosynthesis of **3** was confirmed by comparing its retention time, UV profile and mass spectrometer analysis with an authentic standard.

For the production of **15**, the above reaction was repeated, except SAM was omitted from the reaction. The structure of **15** was proposed based on its retention time, UV profile and observed *m/z* to that of known chaetoviridins and chaetomugilins (Figure S16).

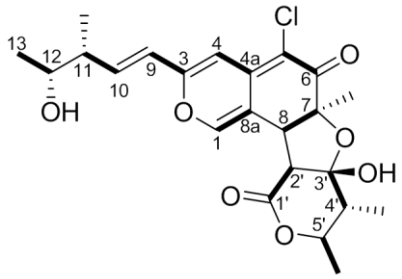
Supplementary Results

Supplementary Table 1. *Primers used in this study*

Primer	Sequence shown 5'–3'
Nseq_07638F	TACCCGTGGAACCATA
Nseq_07638R	TCTGGGATCGACTGTG
cazE_ko_P1	GGGCGAATTGGGTACCATAGTCGTCGTCGTCTTCGGGGTGTCCGC
cazE_ko_P2	CAACAGGACTGTAGCGGGCCAGGGAACCGTCGTGCGTACCCC
cazE_hpfF	GCTACAGTCCTGTTGCGCCTTCCGG
cazE_hpfR	AATGGAACGGTGGAGAAGTTCCCTGGC
cazE_ko_P3	CTCCACCGTTCCATTGCTGGCCGACGCCAACACGCCGTGGG
cazE_ko_P4	ACAAAAGCTGGAGCTGTCCATCCCAACGCGGCAGGGGGTGAATGG
cazE_mt_ver_P0	TGCCGATCAGAGTCTCG
cazE_mt_ver_P5	CAACAGGACTGTAGC
cazE_mt_ver_P6	CTCCACCGTTCCATT
cazE_mt_ver_P7	GGCTTGTACCCAGTGT
cazF_ko_P1	CCTCAACTTCAGCTCGCATCATACG
cazF_ko_P2	GGGAACTTCTCCACCGTTCCATTGGTGACTTGTGTGGCAGGTTGAAC
cazF_hpfF	GTTCAACCTGCCACACAAGTCACCAATGGAACGGTGGAGAAGTTCCC
cazF_hpfR	GGCCAGATCCTCGACCTGCTGGCTACAGTCCTGTTGCGCCTTCCG
cazF_ko_P3	GAAGGCGCAACAGGACTGTAGCCAGCAGGTCGAGGATCTGGCCAAG
cazF_ko_P4	CTTTGTGGCCGCTAGTGTCGTG
cazF_mt_ver_P0	CGTACCAGTCATCACC
cazF_mt_ver_P5	GGGAACTTCTCCACCGTT
cazF_mt_ver_P6	GAAGGCGCAACAGGACTGTAGC
cazF_mt_ver_P7	CGATCTCCAAGACTCG
cazM_ko_P1	ATGATTTCCGTAGCCGACCTCGATTATGCCTCCCGC
cazM_ko_P2	GGCGCAACAGGACTGTAGCCGTTCTCGAGGATTTTCGGCGGGGTTGTATC
cazM_hpfF	GCTACAGTCCTGTTGCGCCTTCCGG
cazM_hpfR	AATGGAACGGTGGAGAAGTTCCCTGGC
cazM_ko_P3	GAACTTCTCCACCGTTCCATTCACCAAGATGAGCCACAACATCGCGGTGCGG
cazM_ko_P4	TCAAAGGGCGTTTCCGTTGCCCGTTCCACGGGGG
cazM_mt_ver_P0	GGGAAGCGAAAGTTGCG
cazM_mt_ver_P5	GGCGCAACAGGACTGTAGC
cazM_mt_ver_P6	GAACTTCTCCACCGTTCCATTC
cazM_mt_ver_P7	GCAGACCTTGCTTCCACA
CazE_F	GACAGCGA**GAATTC**ATGGGTTCCAACCAG
CazE_R	GACGGACG**GCGGCCG**CTCACCCAACAAACTT
CazF_P1_F	TCAACTATCAACTATTAACTATATCGTAATACCATATGGTCGAGGGTGCCAACCA
CazF_P1_R	GGTATCTGGCCCTTCTCCAG
CazF_P2_F	GCATCGGGTCGGTGAAGACA
CazF_P2_R	GTCCAGCGTCGTGGGGTGTA
CazF_P3_F	TCGATCCGGAAGACCTGTTC
CazF_P3_R	GTCGAGTTGGTCATGTGTCG
CazF_P4_F	CCGAGTCACAGGGCTTCAAT
CazF_P4_R	TGTCATTTAAATTAGTGATGGTGATGGTGATGCACCGCCCCTGCAGCTATCTCGA
CazF_ver_1-2F	TCTGATCAAGGTGGTG
CazF_ver_1-2R	CATCAGCGTCTTGTC
CazF_seq1	TGCAACCTGATGCTG
CazF_seq2	CTTCCTCGGTATCTC
CazF_seq3	CCATCAAGGAGATCC
CazF_seq4	GTGGATGAAGGATACG
CazF_seq5	TTACGGAGCGAATACG
CazF_seq6	AGGTATACGCTACCG

EcoRI and NotI restriction recognition sites are underlined

Supplementary Table 2: *Assignments of ^{13}C and coupling constants for [1,2-$^{13}C_2$]acetate-enriched chaetomugilin A (**4**)*

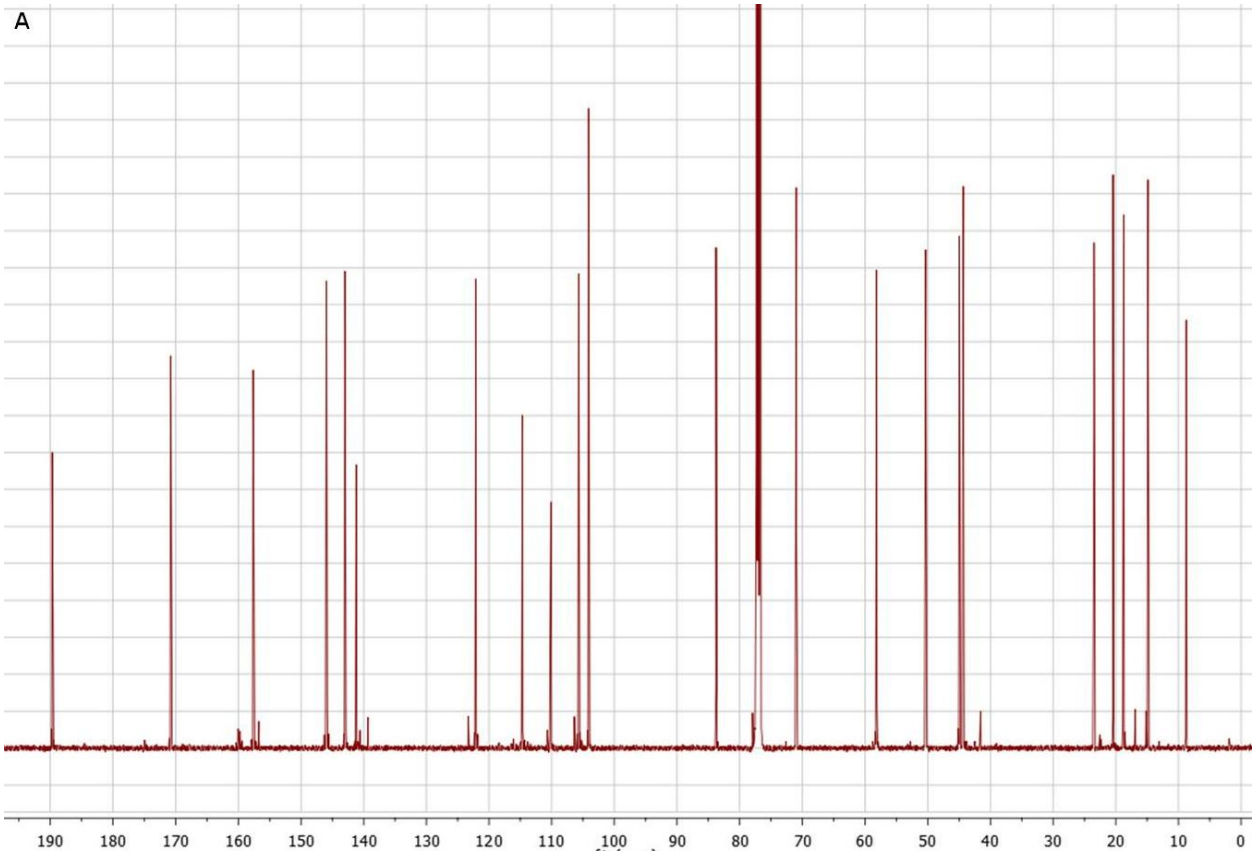


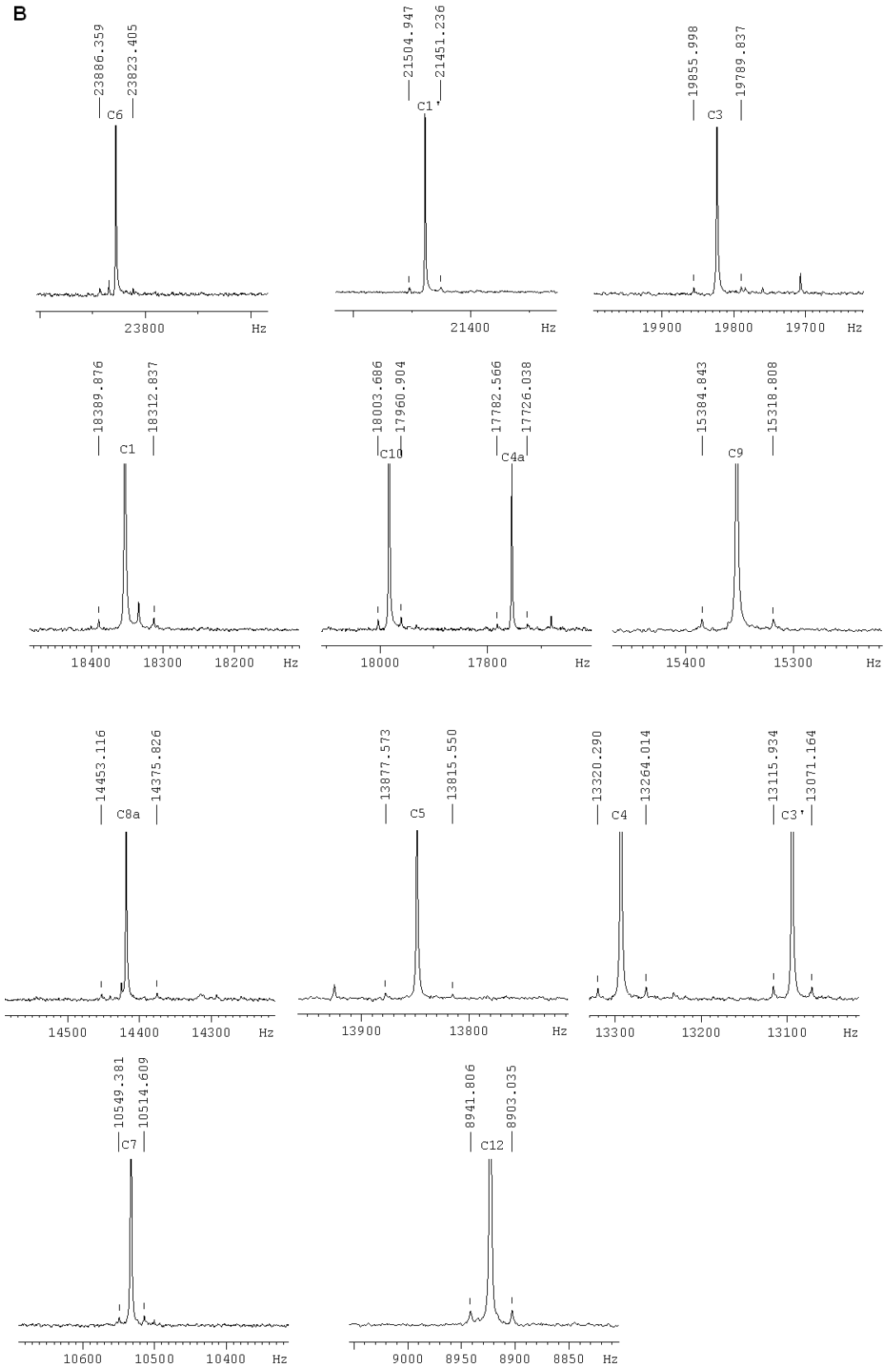
Chaetomugilin A (4)

Carbon[a]	δ_C (ppm)[b][c]	J_{cc} (Hz) [1,2-$^{13}C_2$]acetate
1	145.85	77
2	-	-
3	157.64	66
4	105.72	56
4a	141.20	56
5	110.13	62
6	189.70	62
7	83.77	35
8	50.34	35
8a	114.67	77
9	122.10	66
10	143.01	43
11	44.33	43
12	70.97	39
13	20.45	39
7-Me	23.48	-
11-Me	14.88	-
1’	170.79	54
2’	58.18	54
3’	104.14	45
4’	44.98	45
5’	77.24	41
6’	18.76	41
4’-Me	8.7	-

[a] The numbering scheme for **4** has been adopted from reference[9]; [b] ^{13}C NMR spectral data taken at 125 MHz in $CDCl_3$;[c] δC chemical shifts were compared to reported values[9]; [d] ^{1}H NMR spectral data taken at 500 MHz in $CDCl_3$; [e] δH chemical shifts were compared to reported values[9].

Supplementary Figure 1. *^{13}C NMR spectrum (125 MHz) of chaetomugilin A (**4**) enriched with [1,2-$^{13}C_2$]acetate in $CDCl_3$.* A) Full spectrum; B) Blown up resonances to show the ^{13}C–^{13}C coupling.

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C3
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19800
19700
Hz
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C1
18400
18300
18200
Hz
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C10
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C5
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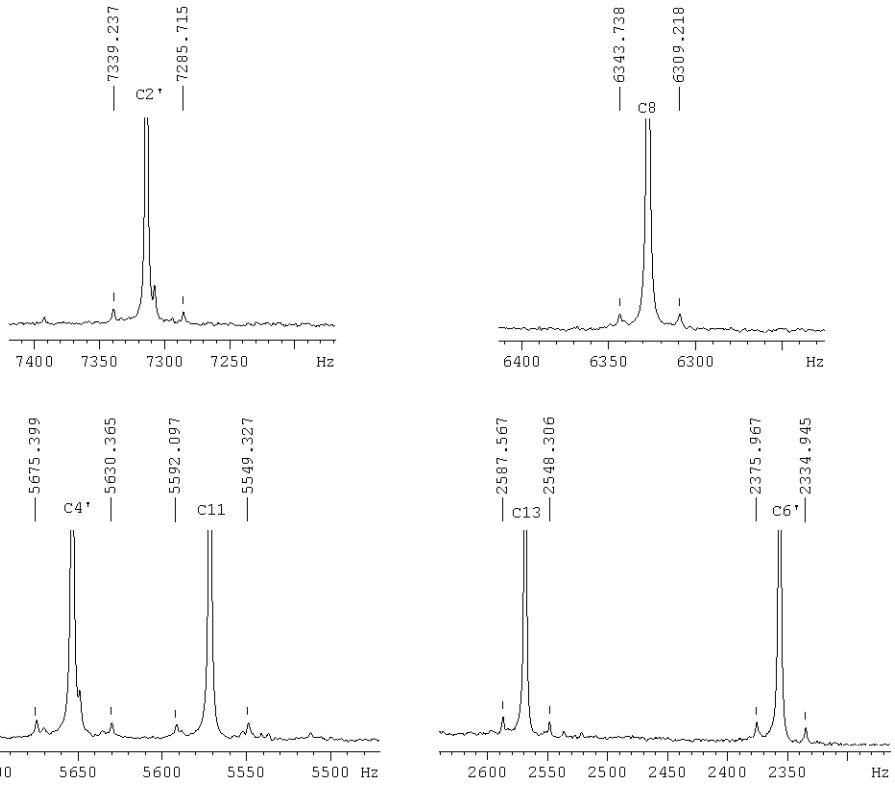
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C13
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2550
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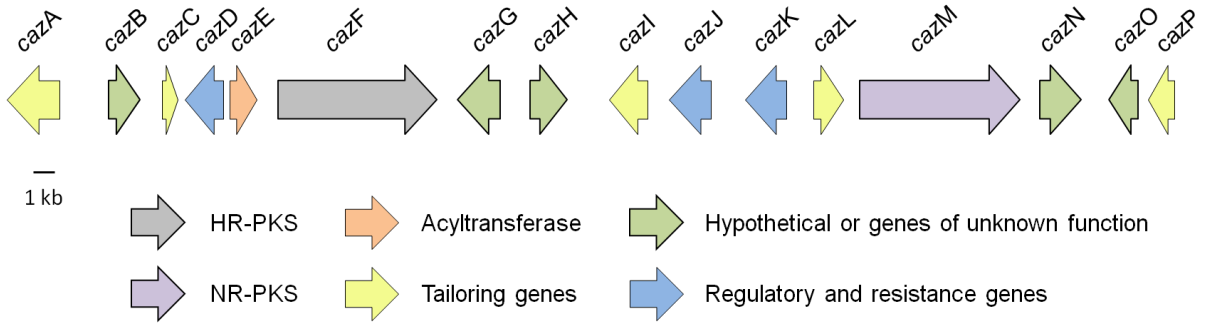
Hz

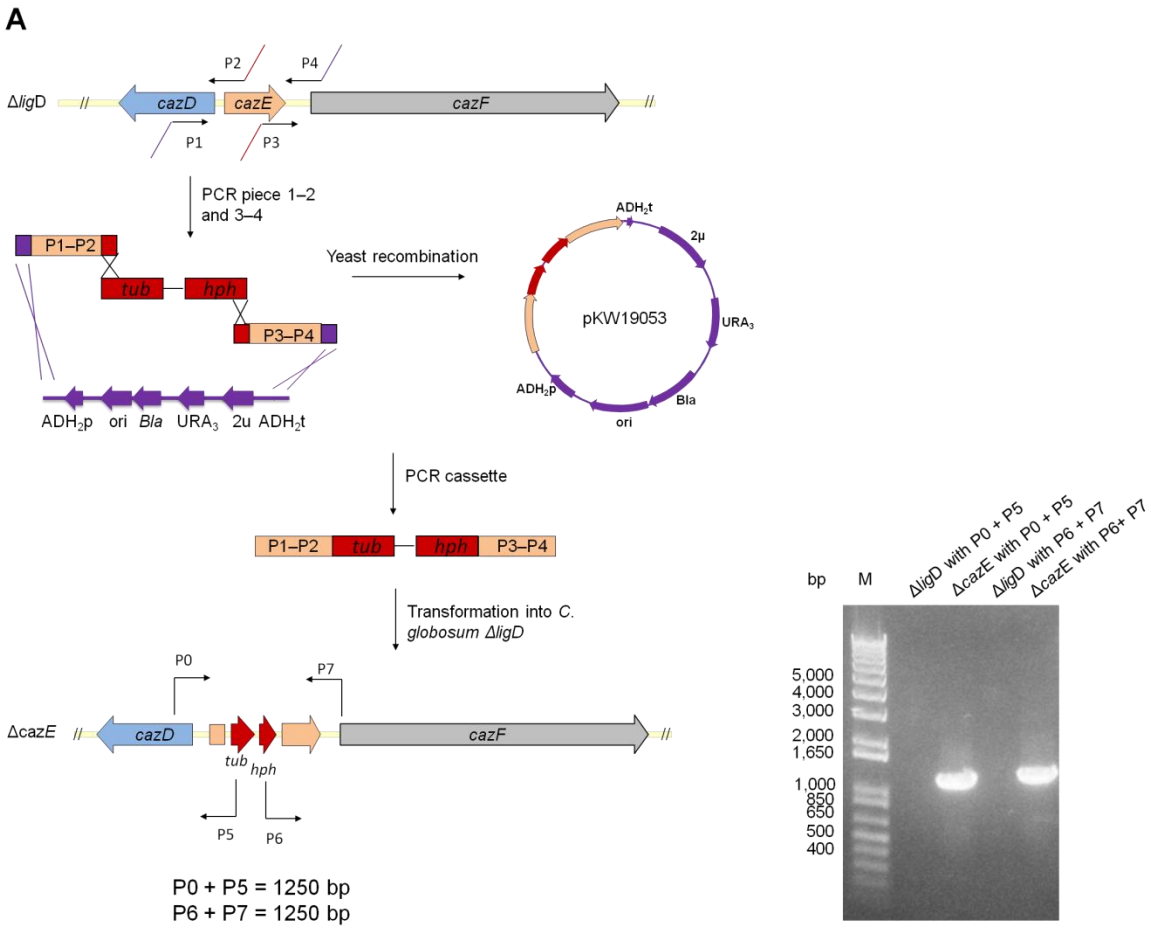
Supplementary Figure 2. *Organization of the caz biosynthetic cluster in Chaetomium globosum CBS 148.51.* The arrows indicate the direction of transcription of an open reading frame.

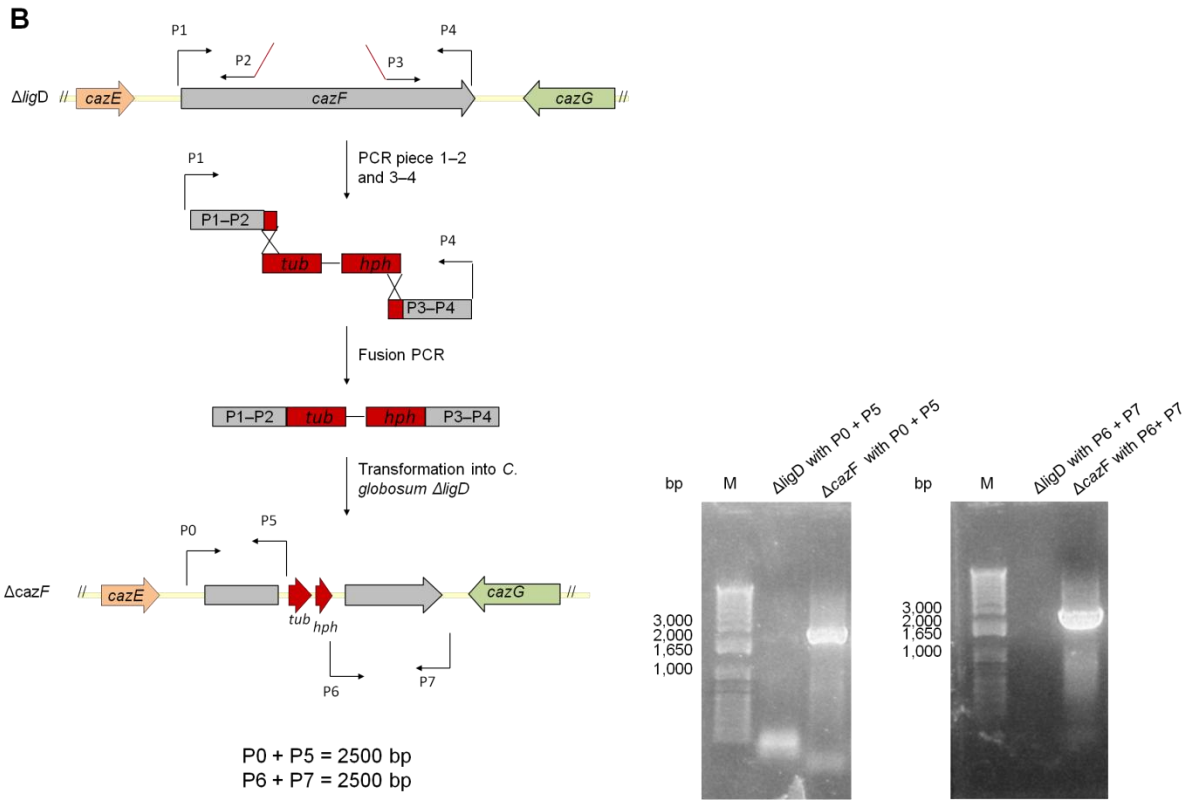


Supplementary Table 3: *Deduced functions of the ORFs in the caz biosynthetic cluster*

Gene Product	Broad designation	Proposed function	Sequence similarity (origin)	Similarity/ identity (%)	Accession number
CazA	CHGG_07633	FAD-dependent oxidoreductase	*Pyrenophora teres f. teres* 0-1	53/68	XP_003298953
CazB	CHGG_07634	Hypothetical protein	*Talaromyces stipitatus* ATCC 10500	39/58	XP_002341174
CazC	CHGG_07635	Glutamine amidotransferase	*Botyrotinia fuckeliana* B05.10	45/62	XP_001550324
CazD	CHGG_07636	Zn(II)2Cys6 transcription factor	*Aspergillus nidulans* FGSC A4	28/54	XP_664394
CazE	CHGG_07637	*O*-acetyltransferase	*Pyrenophora tritici-repentis* Pt-1C-BFP	34/52	XP_001930722
CazF	CHGG_07638	PKS (KS-AT-DH-MT-ER-KR-ACP)	*Aspergillus terreus* NIH2624	68/81	XP_001216280
CazG	CHGG_07639	Hypothetical protein	*Fusarium oxysporum* Fo5176	42/58	EGU88939
CazH	CHGG_07640	Putative transposase	*Sclerotinia scleroiorum* 1980	43/61	XP_001588857
CazI	CHGG_07641	$FADH_2$-dependent halogenase	*Chaetomium chiversii*	61/77	ACM42402
CazJ	CHGG_07642	Zn(II)2Cys6 transcription factor	*Aspergillus nidulans* FGSC A4	50/61	XP_658633
CazK	CHGG_07643	MFS transporter	*Aspergillus fumigates* Af293	65/79	XP_748594
CazL	CHGG_07644	Monooxygenase	*Aspergillus oryzae* RIB40	49/65	XP_001823615
CazM	CHGG_07645 through CHGG_07647	PKS (SAT-KS-AT-PT-MT-ACP-R)	*Aspergillus terreus* NIH2624	70/81	XP_001216282
CazN	CHGG_07648	Polysaccharide lyase	*Leptosphaeria maculans* JN3	56/76	XP_003843726
CazO	CHGG_07649	Glucooligosaccharide oxidase	*Aspergillus nidulans* FGSC A4	47/64	CBF83064
CazP	CHGG_07650	Dehydrogenase	*Aspergillus kawachii* IFO	49/64	GAA86899

Supplementary Figure 3. *Creating disruption cassettes for the inactivation of cazE (A) cazF (B) and cazM (C) and PCR verification of their respective integration in the genome.* Δ*lig*D gDNA was used as a (-) control for all PCR experiments.




B
ΔligD
cazE
cazF
cazG
P1
P2
P3
P4
PCR piece 1–2 and 3–4
P1–P2
tub
hph
P3–P4
Fusion PCR
Transformation into C. globosum ΔligD
ΔcazF
P0
P5
P6
P7
P0 + P5 = 2500 bp
P6 + P7 = 2500 bp
bp
M
ΔligD with P0 + P5
ΔcazF with P0 + P5
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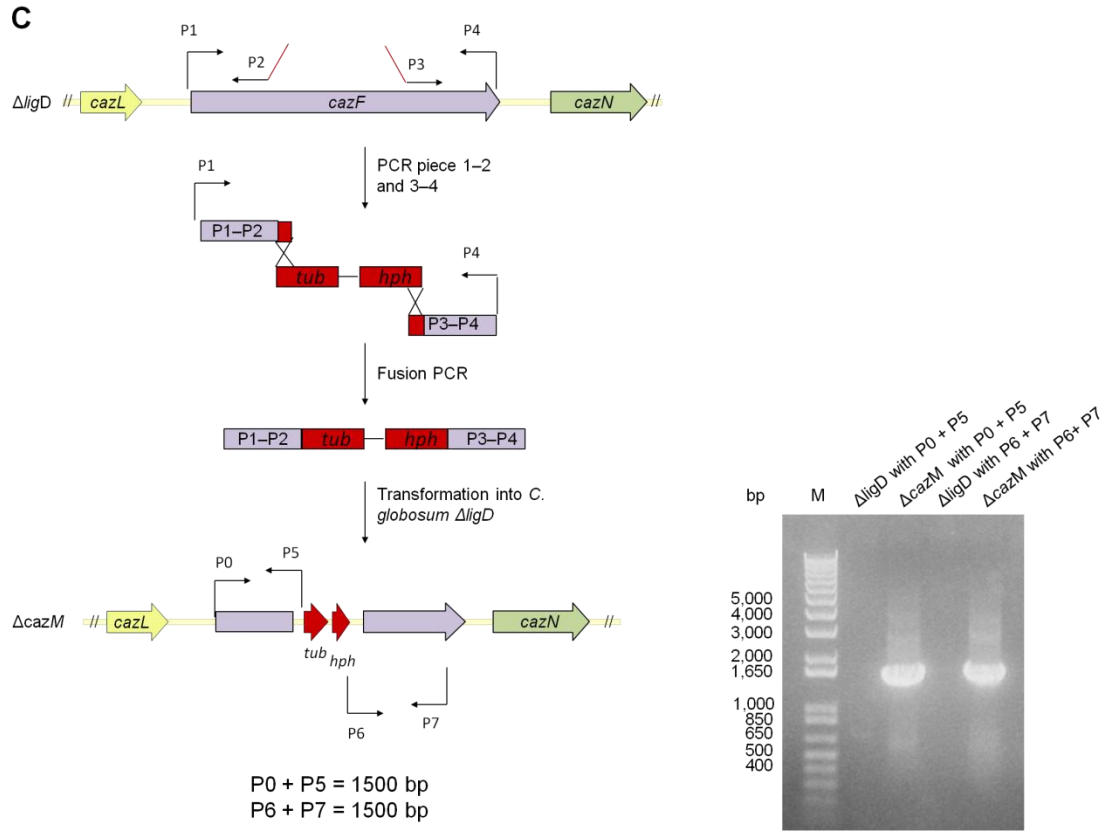
2,000
1,650
1,000
ΔligD with P6 + P7
ΔcazF with P6+ P7


C
ΔligD
cazL
cazF
cazN
P1
P2
P3
P4
PCR piece 1–2 and 3–4
P1–P2
tub
hph
P3–P4
Fusion PCR
Transformation into C. globosum ΔligD
ΔcazM
P0
P5
P6
P7
P0 + P5 = 1500 bp
P6 + P7 = 1500 bp
bp
M
ΔligD with P0 + P5
ΔcazM with P0 + P5
ΔligD with P6 + P7
ΔcazM with P6+ P7
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4,000
3,000
2,000
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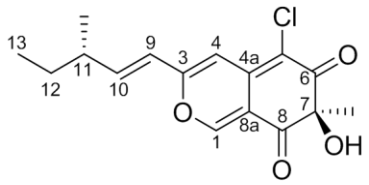
1,000
850
650
500
400

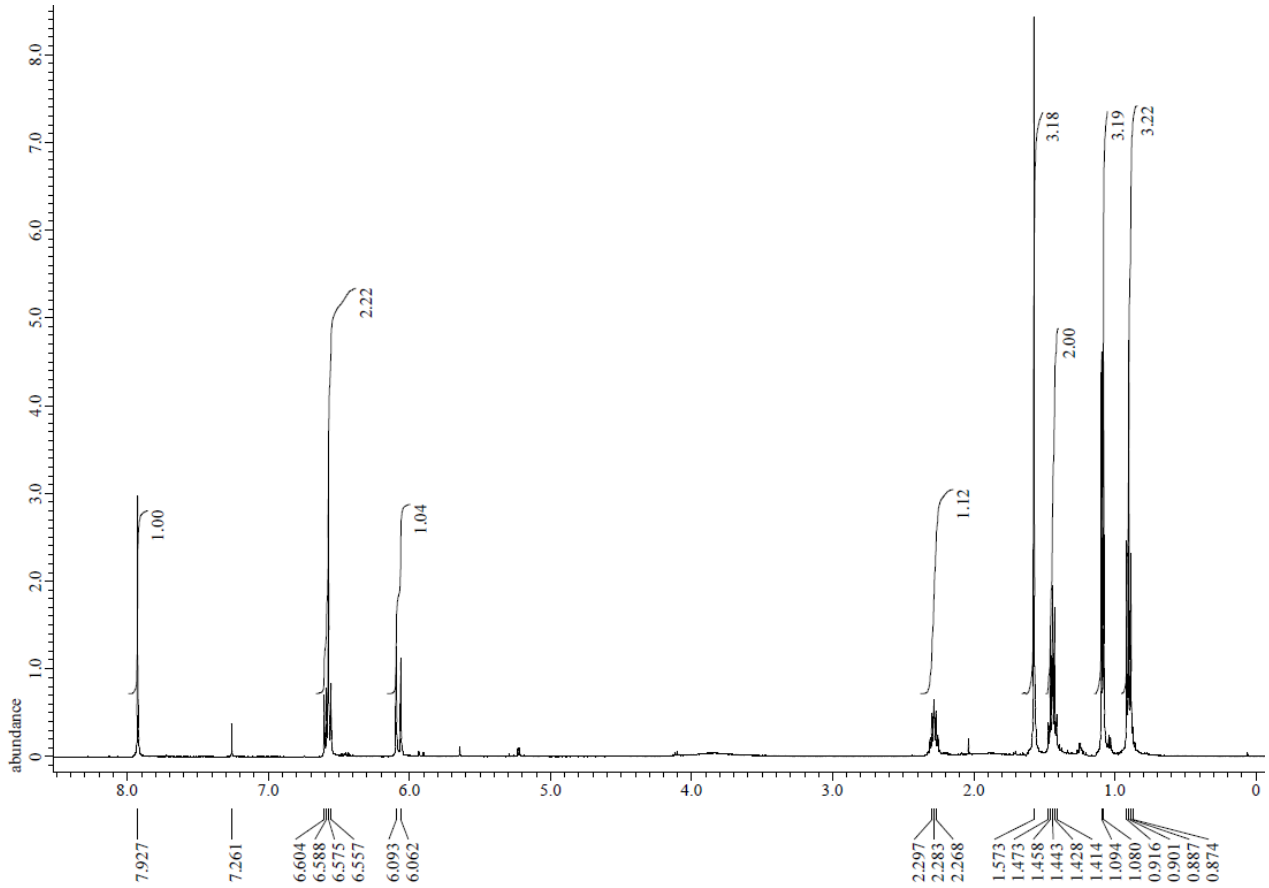
Supplementary Table 4: *Assignments of ^{13}C and ^{1}H NMR data for cazisochromene* ***8.***



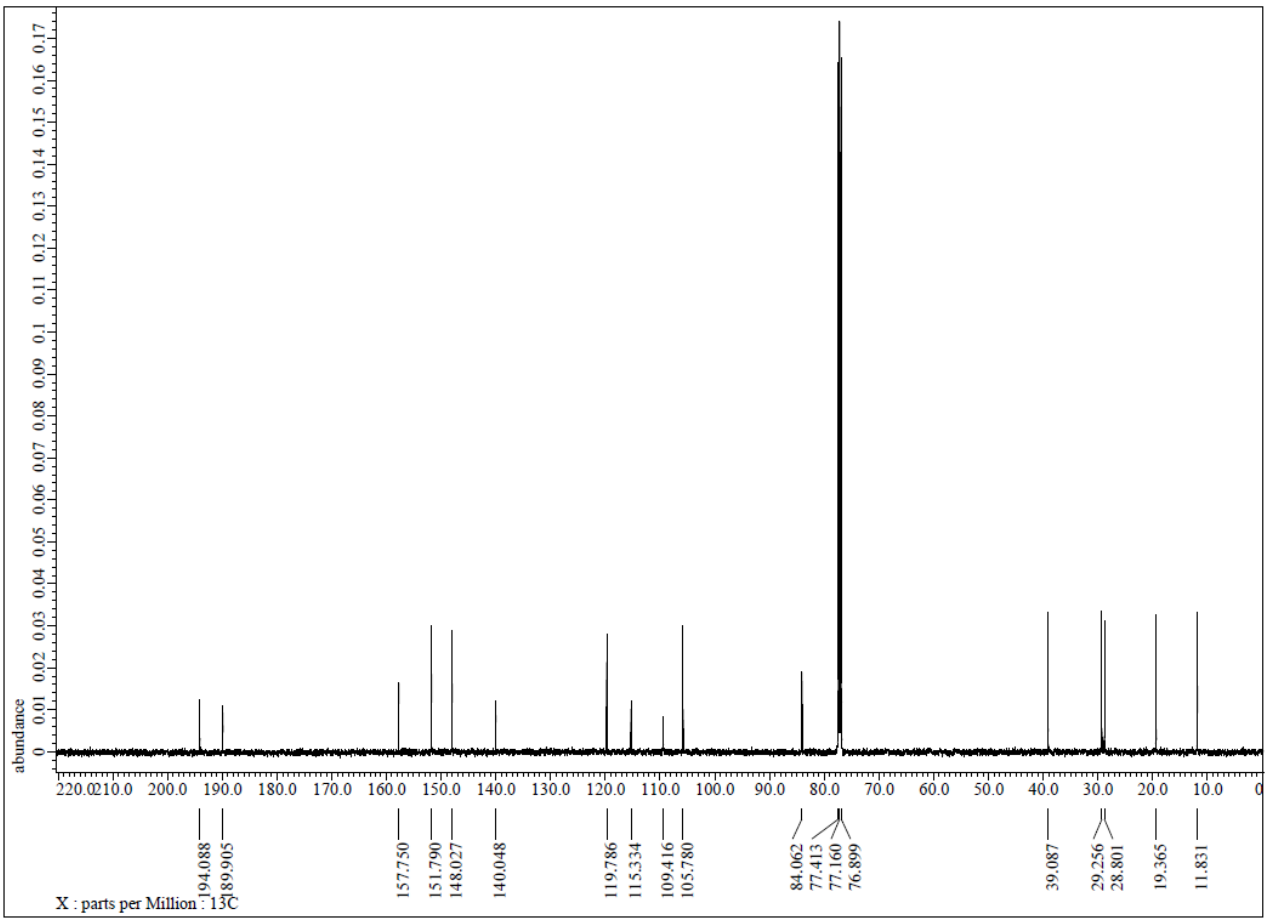
Cazisochromene (8)

Carbon[a]	δCppm[b],	δH(ppm)[c] (J, Hz)	^{1}H-^{13}C-HMBC
1	151.8	7.92 (s)	3, 4a, 8, 8a
2	-	-	
3	157.8	-	
4	105.8	6.57 (s)	3, 5, 8a, 9
4a	140.0	-	
5	109.4	-	
6	189.9	-	
7	84.1	-	
8	194.1	-	
8a	115.3	-	
9	119.8	6.08 (s)	3, 11
10	148.0	6.50 (d,d,15.3, 7.8)	11-Me, 12
11	39.1	2.28 (m)	9, 10, 11-Me, 12,13
12	29.3	1.44 (d,q, 7.4, 7.4)	10, 11, 11-Me, 13
13	11.8	0.91 (t, 7.4)	11, 12
7-Me	28.8	1.57 (s)	6, 7, 8
11-Me	19.4	1.1 (d, 7.4)	10,11,12

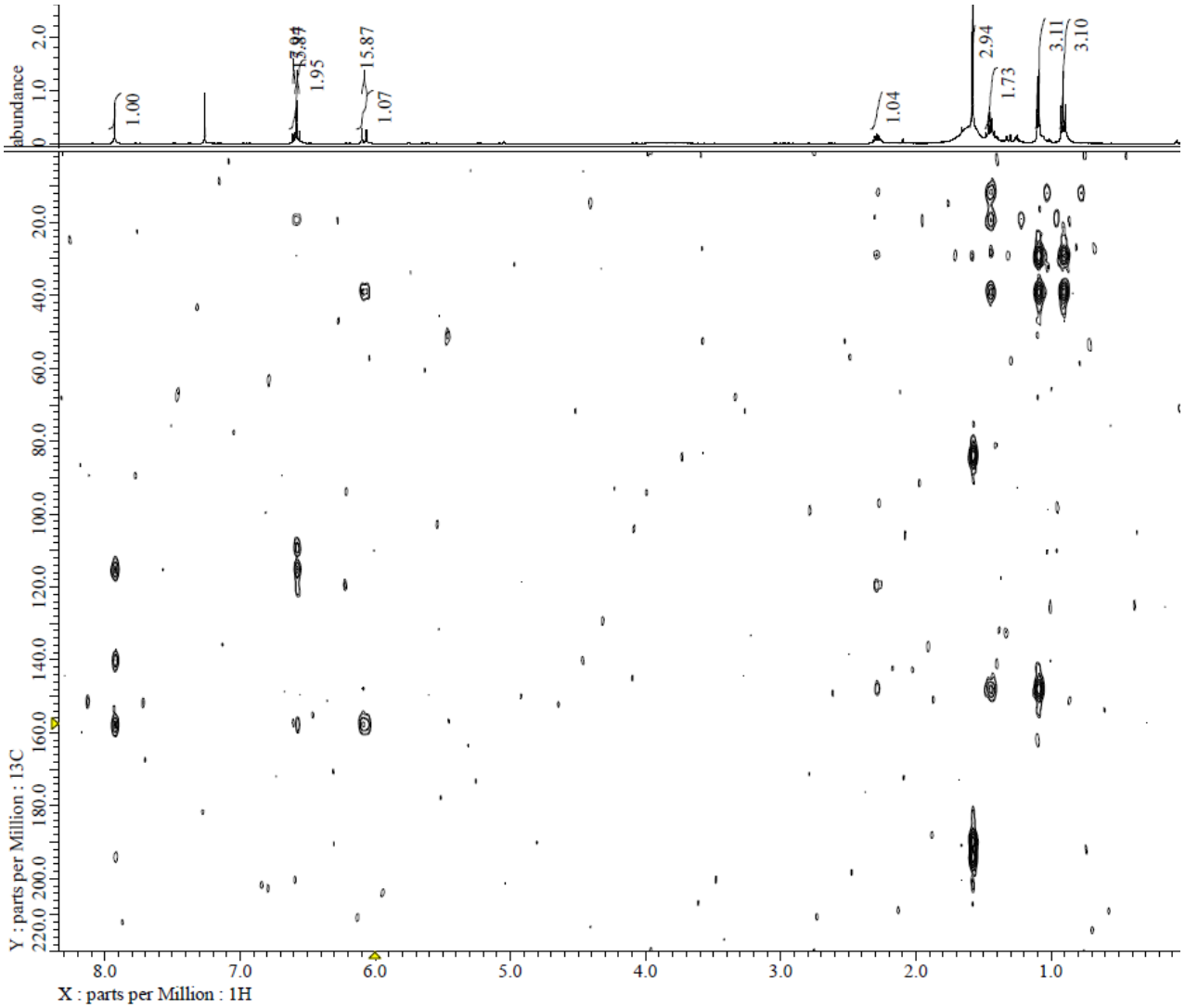
[a] The numbering scheme for intermediate (**8**) has been adopted from reference[9] ; [b] ^{13}C NMR spectral data taken at 125 MHz in $CDCl_3$; [c] ^{1}H NMR spectral data taken at 500 MHz in $CDCl_3$.



Supplementary Figure 4. *^{1}H NMR spectrum (500 MHz) of cazisochromene (**8**) in $CDCl_3$*

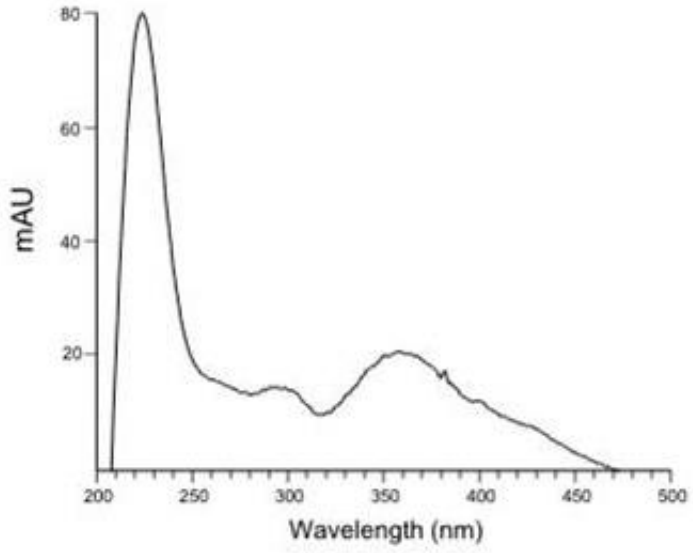


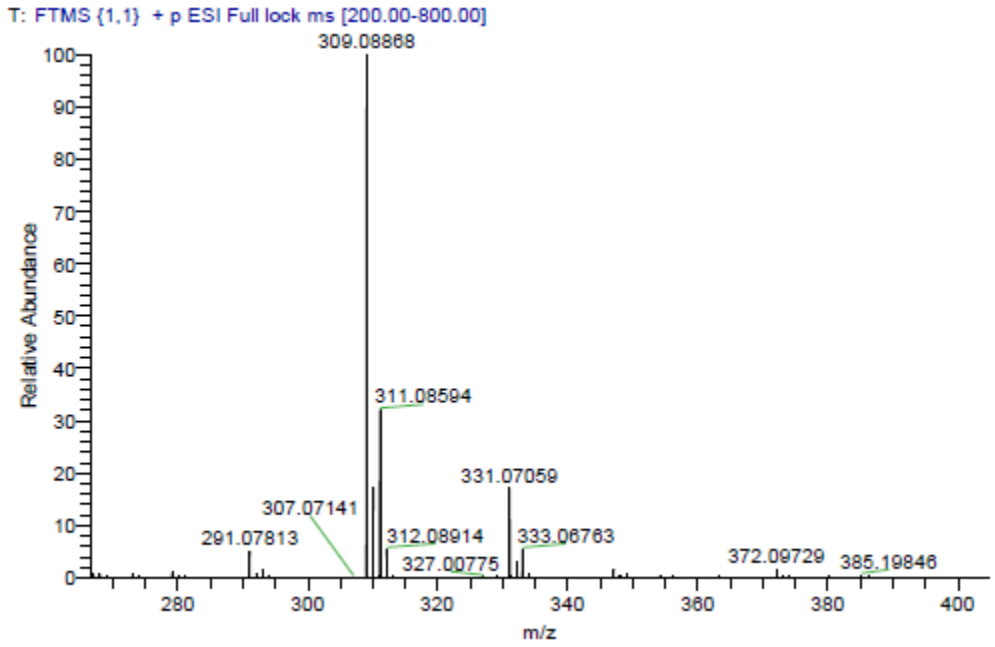
Supplementary Figure 5. *^{13}C NMR spectrum (125 MHz) of cazisochromene (**8**) in $CDCl_3$*



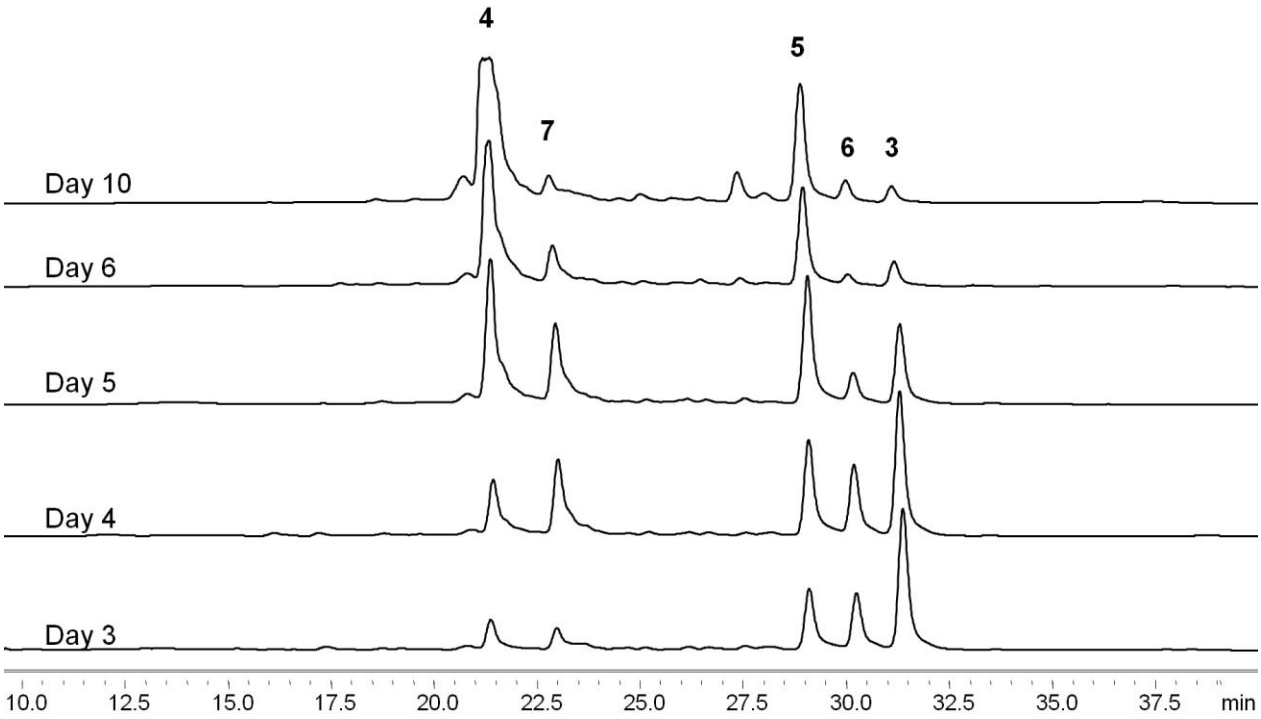
Supplementary Figure 6. *HMBC of cazisochromene (**8**) in $CDCl_3$*

Supplementary Figure 7. *UV profile and mass spectrum [M+H] of cazisochromene* ***8****.*





Supplementary Figure 8. *Time course study of azaphilone metabolites produced by C. globosum.*



Supplementary Figure 9. *Correct DNA sequence of cazF (CHGG_07638).* Introns are indicated by underlined letters and the sequenced gap is highlighted in grey.

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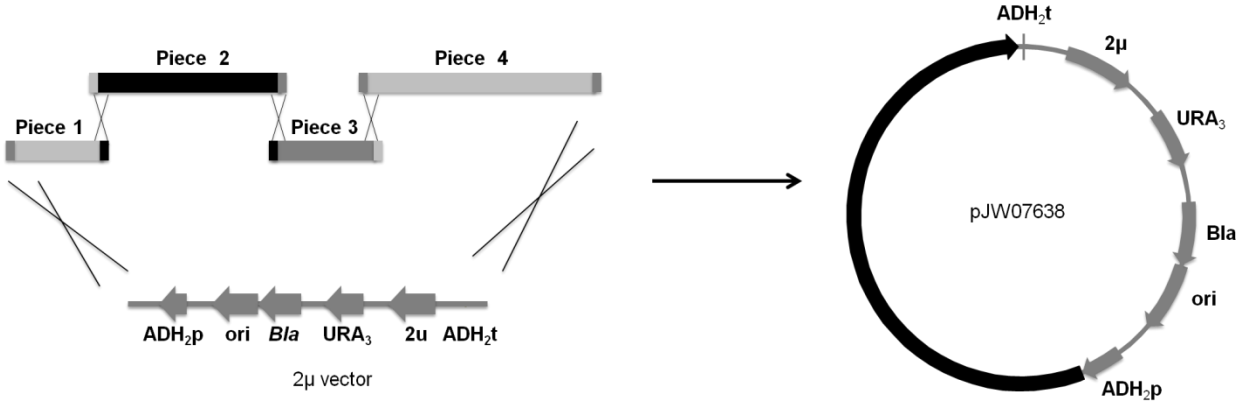
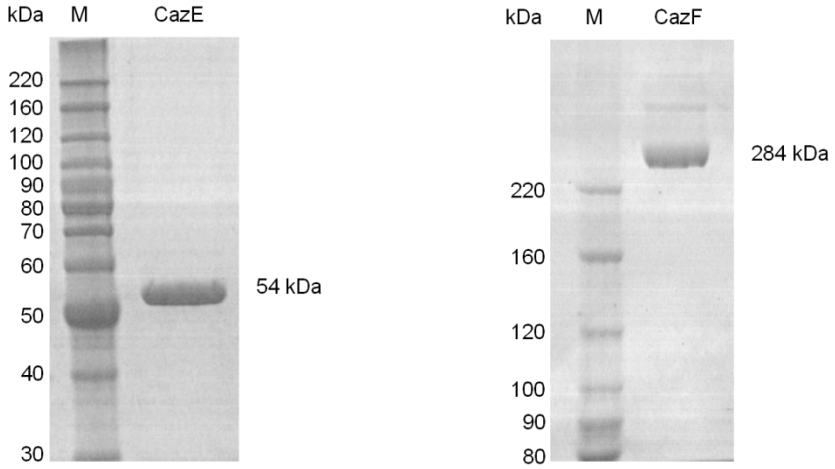
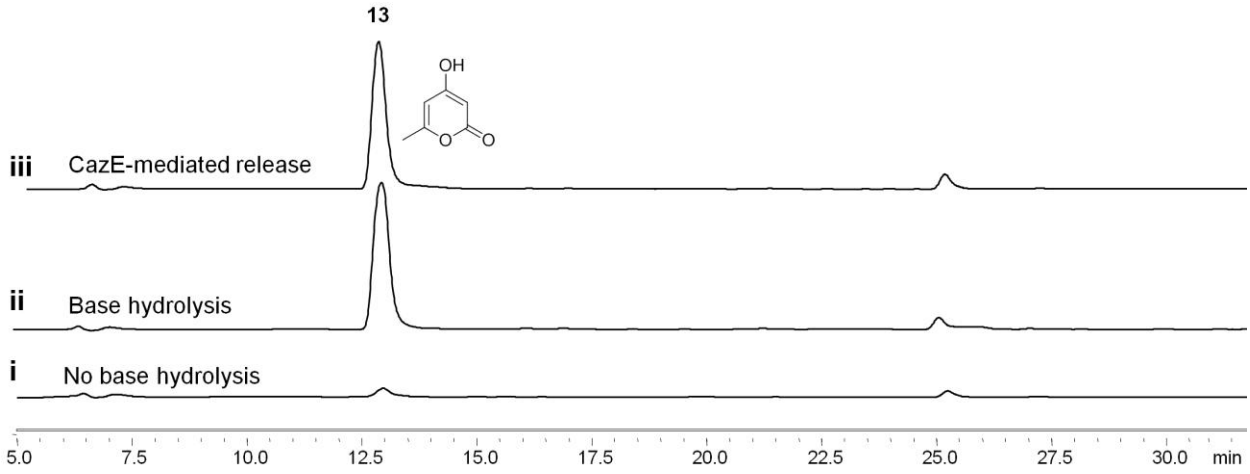
Supplementary Figure 10. *Reconstitution of CazF for expression in S. cerevisiae.* The 7,863 bp intron-free gene was assembled and inserted into the 2μ vector by *in vivo* homologous recombination. Overlapping regions between two flanking segments of CazF ranged from 100-176 bp and overlapping regions between the segments and the 2μ vector were 35 bp.



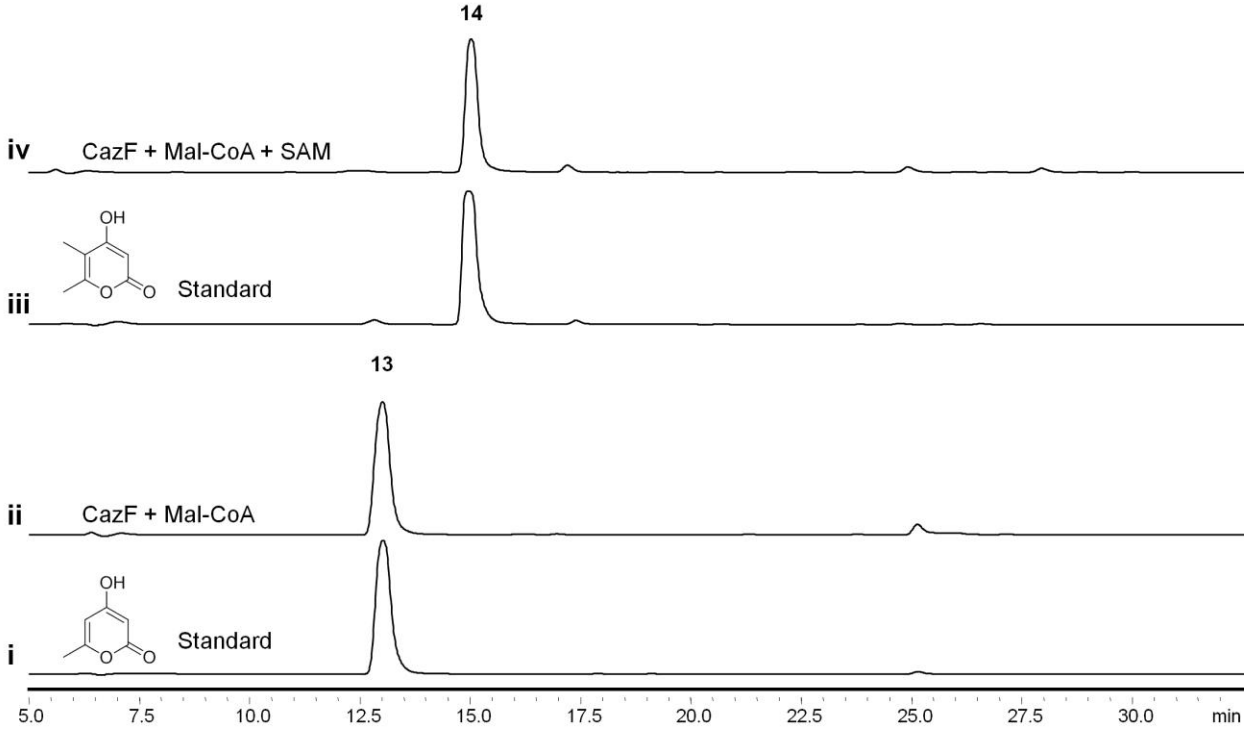
Supplementary Figure 11. *SDS-PAGE of recombinant CazE and CazF.* CazE (54 kDa) was expressed as a N-terminal octahistidyl-tagged protein in *E. coli* BL21(DE3) cells and purified by Ni-NTA agarose affinity resin to yield 52 mg/L. CazF (284 kDa) was expressed as a C-terminal hexahistidyl-tagged protein in *S. cerevisiae* BJ5464-NpgA and purified by Ni-NTA agarose affinity resin at 9.5 mg/L.

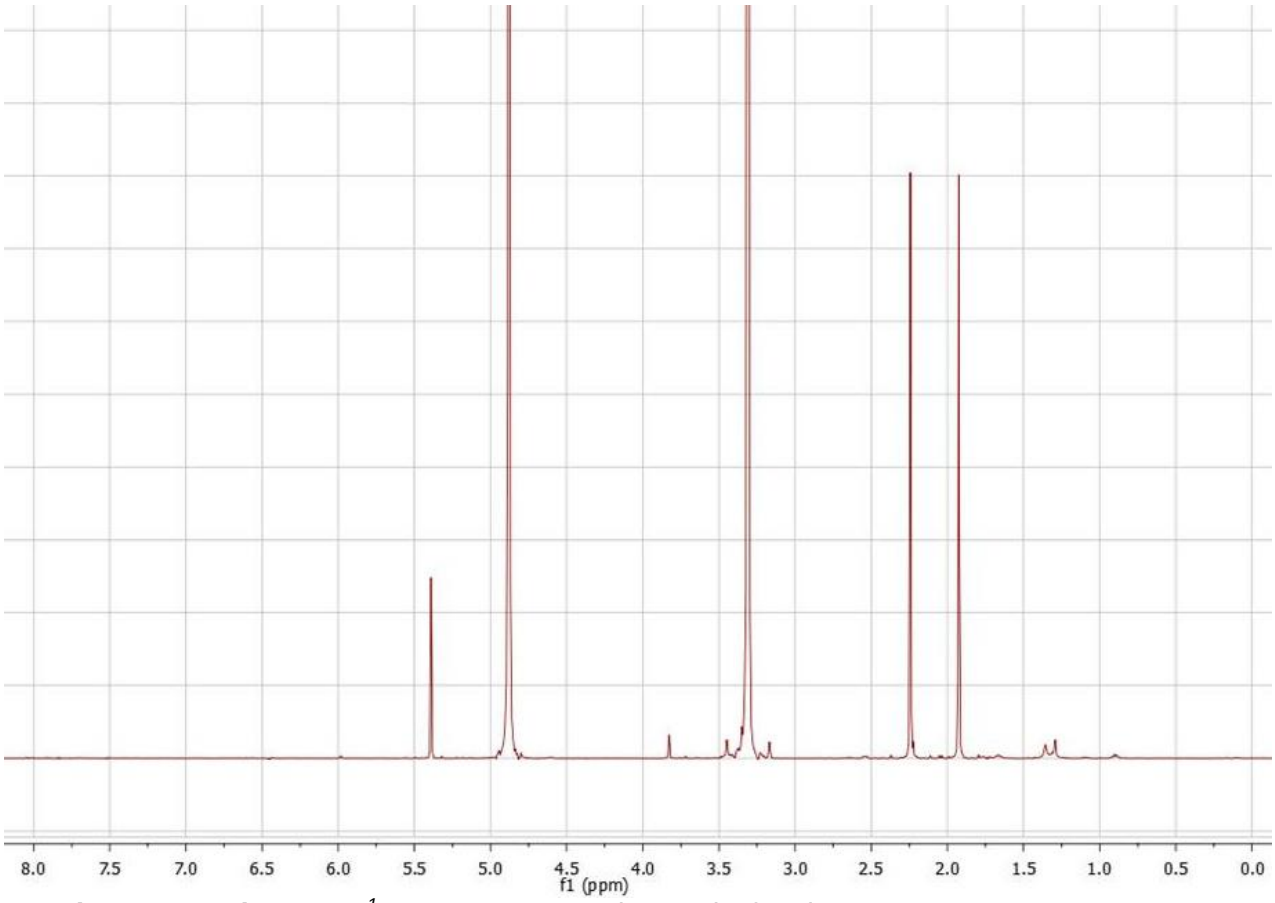


Supplementary Figure 12*. Minimal PKS activity of CazF.* i) Amount of alpha-pyrone **13** observed after the in vitro reaction is extracted with only ethyl acetate; ii) Amount of **13** observed when the in vitro reaction is base hydrolyzed before extraction with ethyl acetate; iii) Amount of **13** observed when CazE is added to the assay and the reaction is extracted by ethyl acetate. HPLC traces are shown in the same scale and observed at λ= 280 nm.



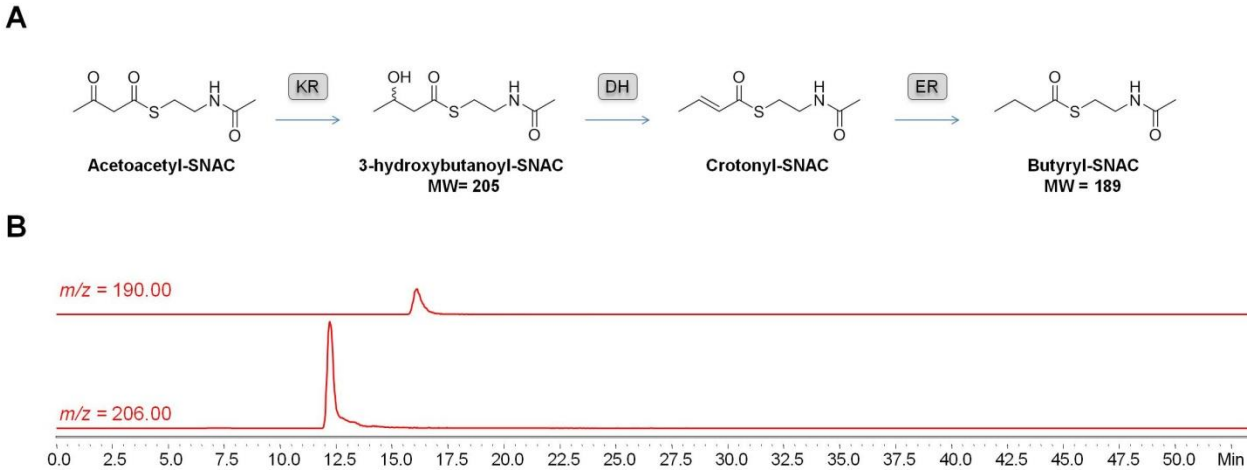
Supplementary Figure 13*. Minimal PKS activity of CazF and the activity of its methyltransferase domain.* i) Synthetic standard of the alpha-pyrone **13**; ii) Compound **13** produced in the CazF in vitro assay with malonyl-CoA; iii) Synthetic standard of the triketide alpha-pyrone **14**; iv) Compound **14** produced in the CazF in vitro assay with malonyl-CoA and SAM. HPLC traces are shown at λ= 280 nm.



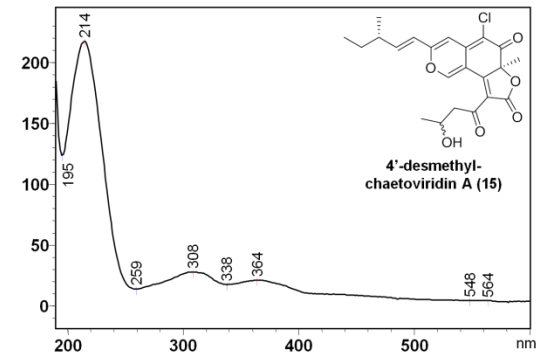


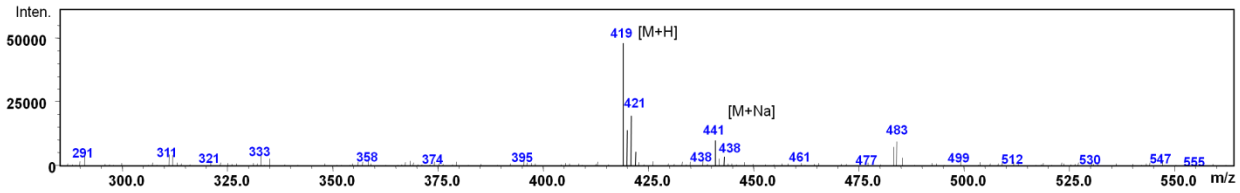
Supplementary Figure 14. *^{1}H NMR spectrum (500 Hz) of **14** in MeOD*

Supplementary Figure 15*. Reduction activity of CazF using acetoacetyl-SNAC as a substrate.* A) Scheme showing the reduction of acetoacetyl-SNAC to butyryl-SNAC using the KR, DH and ER domains of CazF; B) Mass spectrum analysis using selected ion monitoring of 3-hydroxybutanoyl-SNAC and butyryl-SNAC observed in the reduction assay. No *m/z* for crotonyl-SNAC could be detected. All traces are shown in the same scale.



Supplementary Figure 16. *UV profile and mass spectrum of 4'-desmethyl-chaetoviridin A (**15**).*





.Supplementary References

(1) Mizutani, O.; Kudo, Y.; Saito, A.; Matsuura, T.; Inoue, H.; Abe, K.; Gomi, K. *Fungal Genet. Biol.* **2008**, *45*, 878.

(2) Lee, K. K.; Da Silva, N. A.; Kealey, J. T. *Anal. Biochem.* **2009**, *394*, 75.

(3) Keszenman-Pereyra, D.; Lawrence, S.; Twfieg, M. E.; Price, J.; Turner, G. *Curr. Genet.* **2003**, *43*, 186.

(4) Yokoe, H.; Mitsuhashi, C.; Matsuoka, Y.; Yoshimura, T.; Yoshida, M.; Shishido, K. *J. Am. Chem. Soc.* **2011**, *133*, 8854.

(5) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, a Laboratory Manual* Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

(6) Szewczyk, E.; Nayak, T.; Oakley, C. E.; Edgerton, H.; Xiong, Y.; Taheri-Talesh, N.; Osmani, S. A.; Oakley, B. R. *Nat. Protoc.* **2006**, *1*, 3111.

(7) Tsunematsu, Y.; Ichinoseki, S.; Nakazawa, T.; Ishikawa, N.; Noguchi, H.; Hotta, K.; Watanabe, K. *J. Antibiot.* **2012**, *65*, 377.

(8) Jez, J. M.; Ferrer, J. L.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Biochemistry* **2000**, *39*, 890.

(9) Yamada, T.; Doi, M.; Shigeta, H.; Muroga, Y.; Hosoe, S.; Numata, A.; Tanaka, R. *Tetrahedron Lett.* **2008**, *49*, 4192.