Supplementary information

Depsipeptide Aspergillicins Revealed by Chromatin Reader Protein Deletion

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Experimental section

General procedures

Analytical grade chemicals and reagents were supplied from Sigma-Aldrich, Alfa Aesar, Acros Organics and Fischer, unless otherwise stated. Solvents used for HPLC-DAD analyses were HPLC grade, and for UPLC-HRMS analysis were LCMS grade. General molecular biology procedures were performed as standard and molecular biology kits used according to manufacturer's protocols. Analytical PCR was performed using PfuII Ultra high fidelity (Agilent) Expand Long Template PCR (Roche) DNA polymerases.

NMR

NMR experiments were conducted on the following spectrometers: Bruker Avance-500 DCH cryoprobe (^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz), Bruker Avance-600 TCI-F cryoprobe (^{1}H NMR at 500 MHz and ^{13}C NMR at 150 MHz). Chemical shifts were recorded in parts per million (ppm referenced to the appropriate residual solvent peak).

HPLC-DAD

HPLC-DAD for analysis and purification was performed on Gilson GX-271 Liquid Handler with system 322 H2 Pump connected to a 171 Gilson Diode Array Detector and fraction collector. A XBridge BEH C18 XP Column (130 Å, 2.5 µm, 4.6 mm x 100 mm) with XBridge BEH C18 XP VanGuard Cartridge (130 Å, 2.5 µm, 3.9 mm x 5 mm) was used for analytical run with a flow rate of 0.8 mL/min. A XBridge BEH C18 OBD Prep Column (130 Å, 5 µm, 19 mm x 250 mm) with a XBridge BEH C18 Prep Guard Cartridge (130 Å, 5 µm, 19 mm x 10 mm) was used for preparative run with flow rate of 16 mL/min. HPLC grade water with 0.5% formic acid (solvent A) and HPLC grade acetonitrile with 0.5% formic acid (solvent B) were used with the following gradient 0 min, 20% Solvent B; 2 min, 20% Solvent B; 15 min, 95% Solvent B; 20 min, 95% Solvent B; 20 min, 20% Solvent B; 25 min, Solvent B. Data acquisition and procession for the HPLC-DAD were controlled by TRILUTION LC V3.0.

UHPLC-HRMS

UHPLC-HRMS was performed on a Thermo Scientific-Vanquish UHPLC system connected to a Thermo Scientific Q Exactive Orbitrap mass spectrometer in ES^{+} and ES^{-} mode between 200 m/z and 1000 m/z to identify metabolites. A Zorbax Eclipse XDB-C18 column (2.1 × 150 mm, 1.8 µm particle size) was used with a flow rate of 0.2 mL/min for all samples. LCMS grade water with 0.5% formic acid (solvent A) and LCMS grade acetonitrile with 0.5% formic acid (solvent B) were

used with the following gradient 0 min, 20% Solvent B; 2 min, 20% Solvent B; 15 min, 95% Solvent B; 20 min, 95% Solvent B; 20 min, 20% Solvent B; 25 min, Solvent B. Nitrogen was used as the sheath gas. Data acquisition and procession for the UHPLC-MS were controlled by Thermo Scientific Xcalibur software.

Fungal strains and media

Strains used in this study are listed in Supplementary Table S1. They were grown on glucose minimal media (GMM)[1] with additional supplements for auxotrophic strains. Solid and liquid cultures were grown in a light incubator at 30 °C. All strains were maintained as glycerol stocks at −80 °C. Strain TJW149 is used as our wild-type control unless otherwise noted.

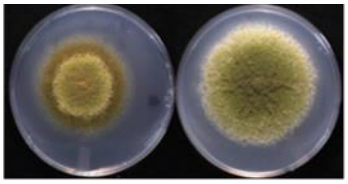


Figure S1. *A. flavus* Δ*sntB* (left) and wild type (right) strains grown on GMM for 6 days at 30 °C.

Table S1. List of strain used in this work.

Strain Name	Genotype	Reference
TJW149	Δ*ku70::A. parasiticus pyrG*	[2]
TJES20.1	Δ*ku70*; *pyrG*-; Δ*argB*::*A. fumigatus pyrG*	[3]
TXZ13	Δ*ku70*; *pyrG*-; Δ*argB*; Δ*sntB::A. flavus argB*	[3]
TXZ14	Δ*ku70:: A. fumigatus pyrG*; *pyrG*-; Δ*argB*; Δ*sntB::A. flavus argB*	[3]
TCG3.1	Δ*ku70*; *pyrG*-; Δ*argB*; Δ*sntB::A. flavus argB;* Δ*agiA::A. fumigatus pyrG*	This study
TCG4.1	Δ*ku70*; *pyrG*-; Δ*argB*; Δ*sntB::A. flavus argB;* Δ*agiB::A. fumigatus pyrG*	This study
TCG5.1	Δ*ku70*; *pyrG*-; Δ*argB*; Δ*sntB::A. flavus argB;* Δ*sidJ::A. fumigatus pyrG*	This Study
TCG6.1	Δ*ku70*; *pyrG*-; Δ*argB*; Δ*sntB::A. flavus argB;* Δ*sidF::A. fumigatus pyrG*	This study

DNA fragment construction for gene disruption

All the DNA constructs were prepared using double joint PCR.[4] 1 to 2 kb of 5′ and 3′ flanking sequence of each gene of interest was amplified using oligonucleotides listed in Table S2 (i.e., 5′-F paired with 5′-R) from RAAS233.2 genomic DNA, with the *pyrG* marker amplified from genomic DNA isolated from *A. fumigatus*. These fragments were then fused together *via* PCR to

generate deletion constructs. PCR amplification was carried out on a C1000TM Thermal Cycler (BioNRad).

Table S2. List of oligonucleotides

Name	Sequence	Use
CG-pyrG-F	TGCTCTTCACCCTCTTCGC	Selectable marker
CG-pyrG-R	CTGTCTGAGAGGAGGCACTG	Selectable marker
CG-agiA-5'-F	GAGTAGCAGGGCTTCTGTTC	Gene deletion
CG-agiA-5'-R	ATTTCAGACCCGCGAAGAGGGTGAAGAGCACTCATCCTGCTTC CAATCG	Gene deletion
CG-agiA-3'-F	GACAGTATAATACAAACAAAGATGCAAGAGGGATGTGTGCCATG TACGAG	Gene deletion
CG-agiA-3'-R	GTGCAGGTGGTGGTACATAA	Gene deletion
CG-agiA-5'-F-int	GCAGCGTTGACTTATTCGC	Gene deletion
CG-agiA-3'-R-int	GCCGCCATTTGTGTTGATAG	Gene deletion
CG-agiB-5'-F	GTGCAGTGAACGGTAGACTAG	Gene deletion
CG-agiB-5'-R	ATTTCAGACCCGCGAAGAGGGTGAAGAGCAGGAGCCCGAATCA AGTCTC	Gene deletion
CG-agiB-3'-F	CATCACGCATCAGTGCCTCCTCTCAGACAGATGGTTGGGAGTC AGGATGC	Gene deletion
CG-agiB-3'-R	GCCAGAAAGCCCTCGGAATAG	Gene deletion
CG-agiB-5'-F-int	GCTTCGGGAGTTCGGATC	Gene deletion
CG-agiB-3'-R-int	GGTGTATCCTTGCCAGTTCAC	Gene deletion
CG-sidJ-5'-F	CACAGCGCGAAAGTACGATG	Gene deletion
CG-sidJ-5'-R	ATTTCAGACCCGCGAAGAGGGTGAAGAGCAGGCGTGAAGGATA TCGCTATG	Gene deletion
CG-sidJ-3'-F	CATCACGCATCAGTGCCTCCTCTCAGACAGCCAGGAGAATCGG GACTATCG	Gene deletion
CG-sidJ-3'-R	CTAGGGCCATAGTATTCTCCG	Gene deletion
CG-sidJ-5'-F-int	CTGTAAAAGCCTCTGTGGCG	Gene deletion
CG-sidJ-3'-R-int	CACCTCGTGTCTGGAATCTG	Gene deletion
CG-sidF-5'-F	GGCATTCAACATAGGGACCG	Gene deletion
CG-sidF-5'-R	ATTTCAGACCCGCGAAGAGGGTGAAGAGCAGCTGTAAAGTCAG AGGGATGC	Gene deletion
CG-sidF-3'-F	CATCACGCATCAGTGCCTCCTCTCAGACAGGCCCTGGTTTATAT CTGAGCAA	Gene deletion
CG-sidF-3'-R	CAGACCACTCAGTGCAATCTC	Gene deletion
CG-sidF-5'-F-int	TGTTGTAGCCTTATCCAAGCG	Gene deletion
CG-sidF-3'-R-int	GCGACGACAATATCGCAATTG	Gene deletion
CG-agiB-Check-F	CGTGCCTTCATTGCACTGA	Check mutant
CG-agiB-Check-R	CCAAATCGGTAACTAAGGCGA	Check mutant
CG-agiB-pur-R	CGTCCTTACAGGTACGACAG	Check mutant
CG-sidJ-Check-F	GTAGCCTGAGATCGGCTTC	Check mutant
CG-sidJ-Check-R	CGTACCAGTATCCAGACGGT	Check mutant
CG-sidJ-pur-R	CCGATTGGGTTGATAAGGAGC	Check mutant
CG-sidJ-Check-F	GATGCCGTTGGGTATGCAAT	Check mutant
CG-sidJ-Check-R	GGAGACCCTCCATCGTAAC	Check mutant
CG-NacT-pur-R	CCACGAGTGCTTCAGCT	Check mutant
PyrG-Check-F	GCATCGGTTGACTACGCT	Check mutant
PyrG-Check-R	CGAGCGTAGTCAACCGAT	Check mutant
CG-actin-semi-F	CCACGTTACCACCTTCAACTC	qPCR
CG-actin-semi-R	GGAGATGCCAGGGTACATGG	qPCR
CG-agiA-semi-F	GCGTGTTTGACCATGTGGTGC	qPCR
CG-agiA-semi-R	GCGCTTCCAAGTAATGTGCGG	qPCR
CG-agiB-semi-F	GCCGCCGTACAAACACATCATATC	qPCR
CG-agiB-semi-R	GGCAATGAACCCTCACCAGAG	qPCR
CG-sidJ-semi-F	CAATCGGGAACCGCTGATCC	qPCR
CG-sidJ-semi-R	CGGATTTGAGTGAGGAGCAGC	qPCR
CG-sidF-semi-F	GGACAGAGCGCTTATGCCAG	qPCR
CG-sidF-semi-R	GCCGAAGGCGACGAATACAA	qPCR

Growth conditions and method of extraction

The *A. flavus* strains were grown on glucose minimal media (GMM), potato dextrose agar (PDA) or potato dextrose broth (PDB) for 14 days at 30 °C, the liquid culture was shaken at 200 rpm. Each 25 mL plate was blended in ethyl acetate (100 mL) and water (50 mL). After two hours, the solid was removed using vacuum filtration and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (2 x 25 mL). The combined organic phases were dried over anhydrous magnesium sulfate and concentrated under reduced pressure. To a liquid PDB culture (200 mL), ethyl acetate (300 mL) was added. The mixture was blended and left soaking for 2 hours. The mycelia were removed by vacuum filtration, the organic layer was removed, and the aqueous layer was extracted with ethyl acetated (2 x 100 mL). The combined organic phases were dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The dried extracts were resuspended in LCMS grade acetonitrile at a concentration of 10 mg/mL and filtered through an Acrodisc syringe filter with a nylon membrane (Pall Corporation) (0.45 µm pore size).

Bioinformatic analysis

Analysis cluster

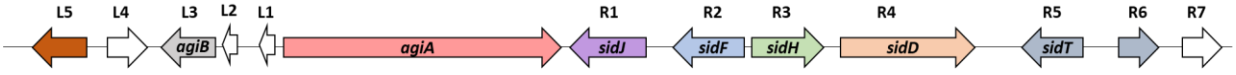


Figure S2. Schematic of the aspergllicin BGC flanked by siderophores genes.

Table S3. Summary of the predicted functions for the genes in the putative aspergillicin cluster. The function was determined using NCBI BLAST in non-redundant database.

Gene	Putative function	Homolog	Gene identity	Query coverage
L5	Hypothetical protein	*A. bombycis*	65%	85%
L4	Lipase 2	*A. nomius* NRRL 13137	88%	99%
		A. arachidicola	84%	99%
L3	*O*-methyl transferase	*A. bombycis*	92%	99%
		A. arachidicola	86%	99%
L2	Hypothetical protein	*A. bombycis*	88%	96%
L1	Hypothetical protein	*A. arachidicola*	92%	98%
NRPS	NRPS	*A. arachidicola*	95%	99%
	(AFLA_010580)	*A. bombycis*	81%	99%
R1	esterase	*A. arachidicola*	99%	99%
R2	Acetylase AceI N-acyltransferase	*A. arachidicola*	98%	96%
R3	enoyl-CoA hydratase	*A. arachidicola*	98%	99%
R4	nonribosomal siderophore peptide synthase Sid2	*A. arachidicola*	96%	99%
R5	ABC transporter sidT	*A. arachidicola*	99%	99%
R6	MFS transporter mirB	*A. arachidicola*	99%	99%
R7	Hypothetical protein	*A. bombycis*	78%	98%
R8	Hypothetical protein	*A. bombycis*	78%	99%

Artemis comparison tool (ACT) analysis

ACT[5] was used to compare the aspergillicin BGC from *A. flavus.* to the genome of *A. fumigatus*, *A. oryzae*, *A. arachhidicola* and *A. nidulans*. The red and blue lines indicate homologous genes with identity above 75%.

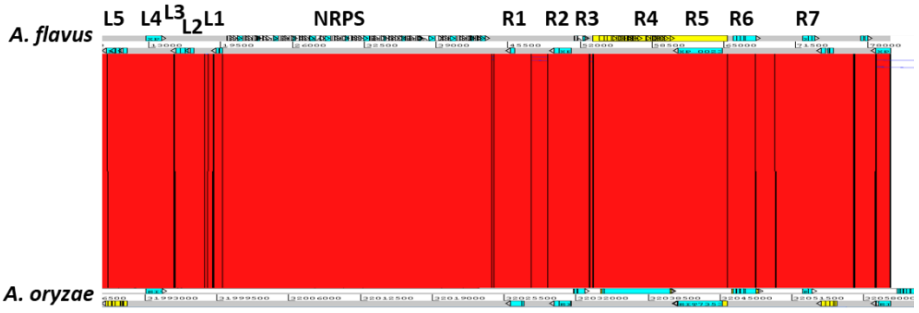


Figure S3. ACT analysis of the aspergillicin BGC from *A. flavus* genome *vs A. oryzae*.

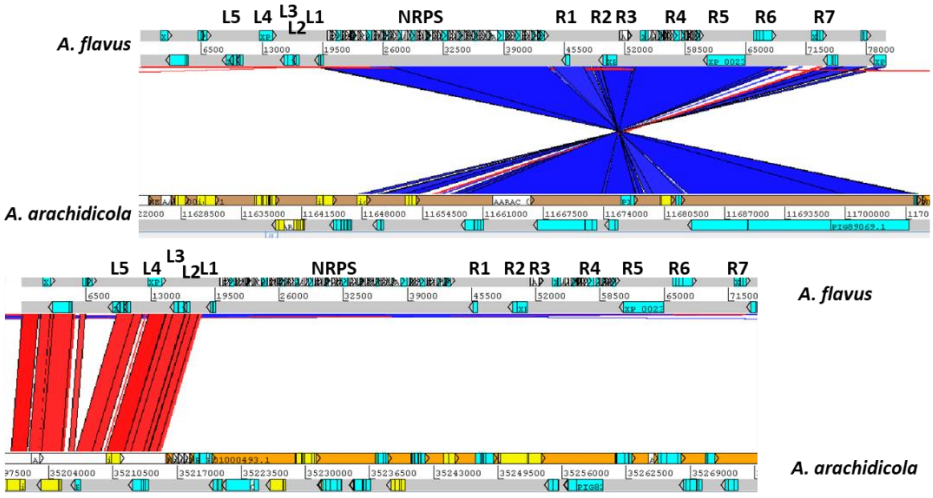


Figure S4. ACT analysis of the aspergillicin BGC from *A. flavus* genome *vs A. arachidicola*. In *A. arachidicola* the BGC is split across two contigs.

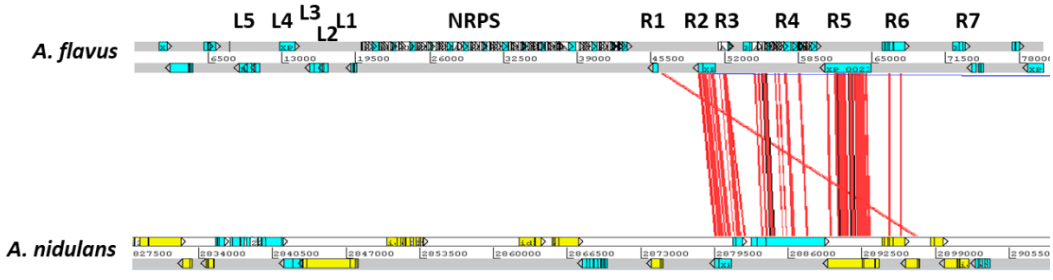


Figure S5. ACT analysis of the aspergillicin BGC from *A. flavus* genome *vs A. nidulans*.

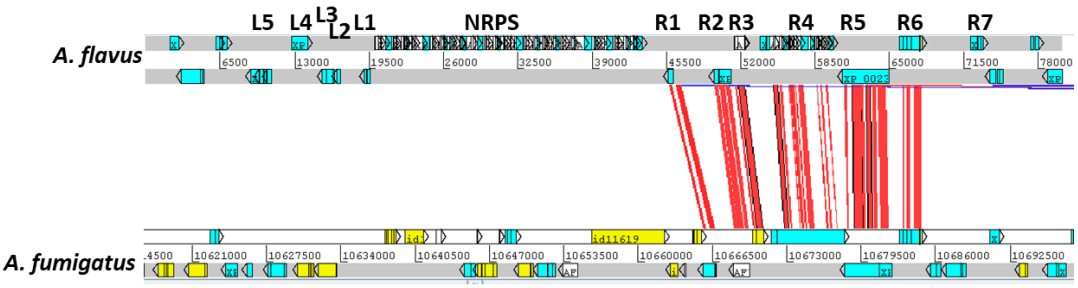


Figure S6. ACT analysis of the aspergillicin BGC from *A. flavus* genome *vs A. fumigatus*.

Multigene BLAST

Multigene BLAST[6] analysis identified that the siderophore BGC is fairly conserved across many fungi. But the NRPS *agiA* is unique to *A. flavus* and closely related species.

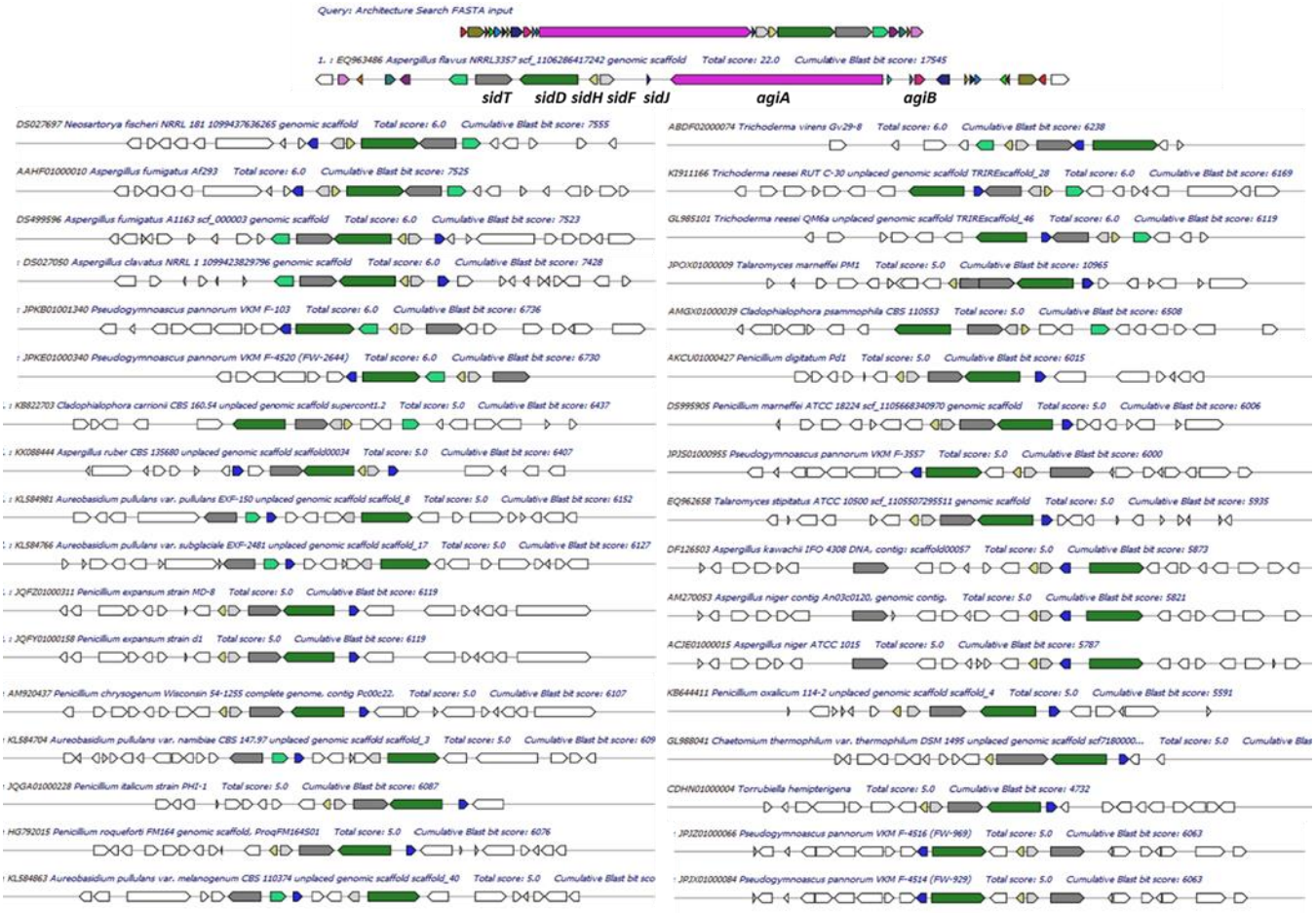


Figure S7. Multigene BLAST analysis of the aspergillicin BGC. The siderophore genes can be identified across different fungi.

Analysis of condensation domain

Multiple sequence alignment using Clustal Omega of the condensation domains of AgiA (AFLA_010580) revealed the canonical HHxxxDG motif.

```
CLUSTAL O(1.2.4) multiple sequence alignment


C5      --VRQSFAQGRLWFLDQLHPGSTWYLMPFGLRIQGD-LHLDALEAAVSAIEERHETLRTT    57
C1      ---APSVMQEEMIVSTIADPSHKSYFETYHFSAQGI-VDPDQLNGAIHAVARKHAVLRSV    56
C3      DIIPCTPMQRALLYEGIADHESRSYVTCRIWRIPTDQAICSQIEGAVKSLIQRHGILRTV    60
C2      AIYPCTPQQEGLIQTSLH-GDKSAYFATITVHLGDS-LNLRTFHAAWNRLVFGCDMLRTA    58
C4      -VMPCTPFQEGVLSSNDESGSSAGYLAHMTVGLGKE-IDVEALKYAWQETVDHEDMLRTT    58
C6      -LMPCTPFQEGVLSNSLAVPGDSGYLSVVRLGLQSQ-LDTKAMRLAWQKVVEREETLRTA    58
             :  *  :           *.                :. *         **:.

C5      FEHRDGENVQVVHPFAH-RQLRVVEVPPAVDEEGLLGALKE-------------------    97
C1      FVHPTEFDSTNV---------QIAVLDQA-YTLHRAKLLSLQ--------------PIGE    92
C3      FHIDPEVGPLA-------LVLRDTQSPTA-SAVGHVKVKDHTEMEERV-T------SLLC    105
C2      FVSFSEVQHPP---VSESNILQVVLSQSA-EDVRRLVSLDNRD----------------I    98
C4      FIPADMDMTDIRGLGQGSSLLQVVLYPES-PQAGRVKTMKTVSTPATPNAALPSYPSLQG    117
C6      FIPVAEDLSSACIT--SSTFWQCIFNINS-REVQRLLCIEGRN------SG-----VDRS    104
        *                    :     :         .

C5      ---EQSTPFDLQVHPGWR--------PL--VLRQNKRSHILSIVIHHIICDGNSVAVLLK    144
C1      EIRFGILPLESIGTDEWDGVMPWKFSLVVC---EREQKSYITVRVHHALLDGNSARALLE    149
C3      NPDYGNPMKQAFYV------------RI--VHAADGSGARLIWLLHHSLIDANSQDLLLS    151
C2      AFQFGVVPLSAGI-----------------SQGSVNEPWRLHLKIHHALYDEAFLSRIVA    141
C4      KAQQGHIPVAALVAI--------------GSQDGEGQECTLLITNHHALYDEAYLSLLLK    163
C6      ALGFGHIPVSLILTDV----------PTVCKARGV-GSTQLELTIHHALYDEAYFRWIIH    153
                                           :    ** : *     ::

C5      ELSTFYSAALHGKPIHA-QLPPLPIQYRDFSAWESQKEQR----VEHDRQLKYWIEKLTG    199
C1      LVQQEMLNPG------------AVLKGSDFFSLVRQPLK-----RDWKQDETLLRERLAQ    192
C3      ELTQILVDG------CP---TKSLIPRPSFGSFARYVTSD---SRADHSHGKFWSETLNG    199
C2      ELCIVYEALEMESSEAA------LPPSRPFSAFVESLCN-----DDPEVSKGFWKKYMHE    190
C4      DLSGRYRSIACNE-VVP---QVPEDQRIPFSTYVRFVHSKLGTAPSTSTAGKFWKSYLAD    219
C6      ELSREYHKARLAKDYVPLRAPQTSMNRIPFSIFVSQLQA-----MPKESATSFWKSYLNG    208
         :                           *                          . :

C5      SK--PAE--FIC--DKRRPQAPSRQAIFEE--VRIDGAMYDQLRQYCKQHQLTPFIVLLA    251
C1      IETSPILSPGPVANGD--LRVGEVTREVSISSDIW----------LPDRPAVPARLLRL    239
C3      VQ--PKS--LPLSLMSSSP-INAA-----AVVVEQACN-----LAILSENCISPAALVSL    244
C2      VA--PAT--WPVASGIRRMREENGQDVEMTVVKSWTGNA----VALGQKFQATPASIVRA    242
C4      AT--PST--WPLPHGMQSS-ITSVKS-PETAVLEWTGNL----RAAASKVQVTAAAIARA    269
C6      AP--AAC--WPVARGLESGRITEIDE-FSSRSLIWKGNM----HNLAGARGVTPAAISRA    259
                                                                 :

C5      VFRATHYRLTREADATIGTPIANRGRE------ELHDIIGLFVNVQCIRLKVD   298
C1      ALGMTISVFRNSDDSLFLEITSARSRL----FPKDQQVLGP------------   276
C3      AWSLVLSEILDTDDVTHGMLFSGRQLP---VD-GVADIIGPCISTVPIRTHL-   292
C2      ALALALAQYSQTDDVVFGEVSSGRFDH-------DRFTLGPCLATHPVRIQI-   287
C4      ALALTVAEHTNVTDVVLGEVSKGRPDIRGPGDARARFITGPCATTHPVRIR--   320
C6      AVALVVAEHSGVEDIVLGEVSSGRSIT----DGAAGFVAGPCISTHPIRIRM-   307
        .   .        *         *              *
```

Figure S8. Multiple sequence aligment done using Clustal Omega. Red box highiligts conserved motif.

Analysis of the thioesterase (TE) domains

Phylogenetic analysis of the *agiA* TE domain was done using Clustal Omega and iTOL with defaults parameters (Figure S9). The TE domain is clearly distinct from condensation domains. There is not a clear distinction among bacterial TE domains in PKS or NRPS genes. In contrast, fungal TE domains in PKS genes are clustered together. Not many TE domains from fungal NRPS are available, but agiA-TE is very closely related to another fungal NRPS-TE involved in the biosynthesis of penicillin. Interestingly, the two fungal NRPS-TE clustered more closely to the bacterial TE domains than the fungal PKS TE domains.

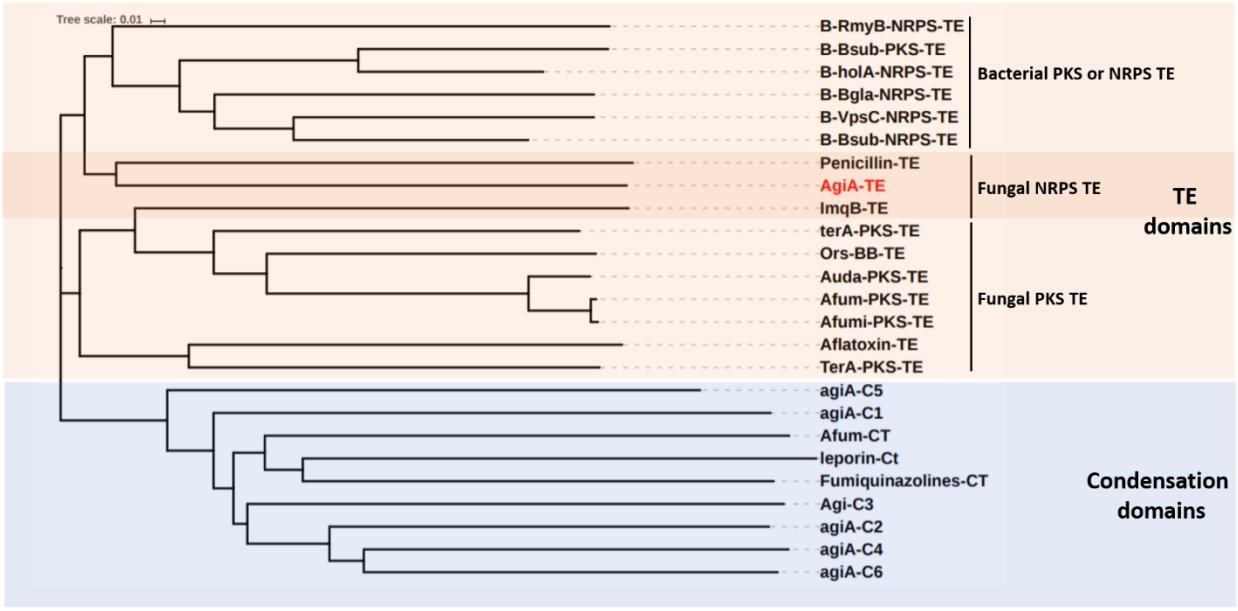


Figure S9. Phylogenetic tree for the TE domains (orange) and C domains (blue). Dark orange highlights fungal NRPS-TE domains. The TE domains is divided in bacterial TEs, fungal NRPS-TE and fungal PKS-TE domains.

Fungal transformation

A fresh spore suspension (2 x 10^6 spores/mL) of TXZ13 was used to inoculate 500 mL of sterile GMM and shaken at 280 rpm and 28 °C until the formation of young germlings (~13 hours). The germlings were centrifuged at 8000 rpm for 15 mins at 4 °C. The supernatant was discarded and 50 mL of sterile dH_2O water was added to the germlings. They were spin at 8000 rpm for 15 mins at 4 °C. The supernatant was removed and the germlings were transferred to 10 mL of osmotic medium (1.2 M $MgSO_4$, 10 mM NAPB*), adjusted to pH 5.8 with 1 M Na_2HPO_4) with 30 mg of Lysing Enzymes (Sigma) and 20 mg of Yatalase (TaKaRa). The germlings were then shaken at 80 rpm and 28 °C, until protoplasts were obtained (approx. 3 h). The protoplast solution (10 mL) were transferred to a 30 mL cortex tube and very gently overlaid with 10 mL of trapping buffer

(0.6 M sorbitol and 0.1 M Tris-HCI, pH 7.0). After centrifugation in the HB-4 (or Beckmann equivalent) rotor at 5000 rpm for 15 min at 4 °C, the protoplasts were removed from the interface and transferred to a 15 mL sterile tube. The protoplasts were diluted with one volume of STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCI, pH 7.5) and centrifuged at 6000 rpm for 10 min at 4 °C. The supernatant was removed, and the protoplasts were resuspended in STC (1-2 mL) and stored on ice. The transforming DNA (e.g. specific gene disruption construct) was diluted with STC (10 µg/100 µL) in a 15 mL falcon tube. The protoplast was added to the DNA (100 µL) and incubated on ice for 50 minutes. Then 1.25 mL of PEG solution (60% PEG 4000, 50 mM $CaCl^2$, 50 mM 10 mM Tris-HCI, pH 7.5) was added, mixed gently and incubated at room temperature for 20 minutes. Next 5 mL of STC was added to the mixture, gently mixed and the entirety plated (500 µL) on GMM media containing sorbitol (1.2 M). Resulting transformants were subcultured twice on selective media and confirmed by Southern and PCR.

*NaPB: 2M phosphate buffer = 90.9 g Na_2HPO_4 and 163.4 g NaH_2PO_4 per liter, with pH 6.5.

Transformant confirmation

The transformants were confirmed using Southern analysis for the Δ*agiA* strain (Figure S10) and PCR analysis for Δ*agiB*, Δ*sidJ* and Δ*agiF* (Figure S11). Primers used are listed on Table S3.

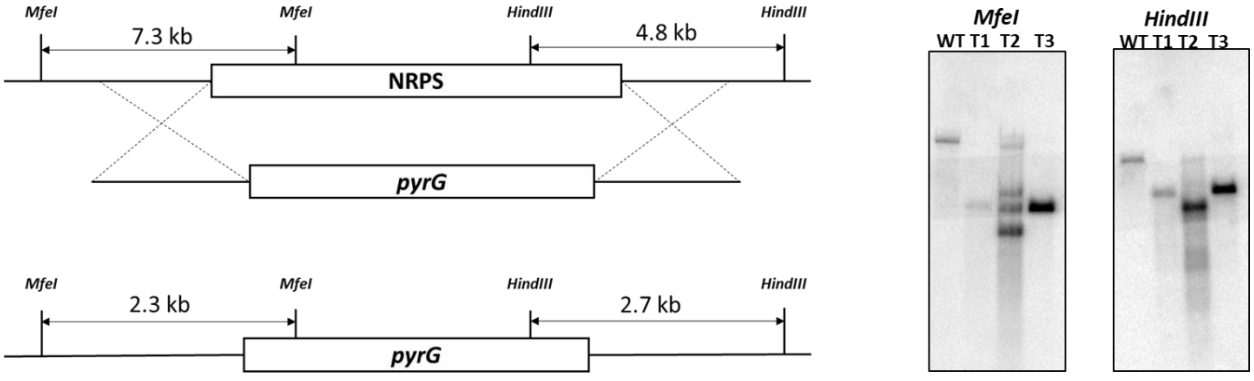


Figure S10. Schematic of the expected product from digestion using *MfeI* and *HindIII* (left). Southern results for Δ*agiA*.

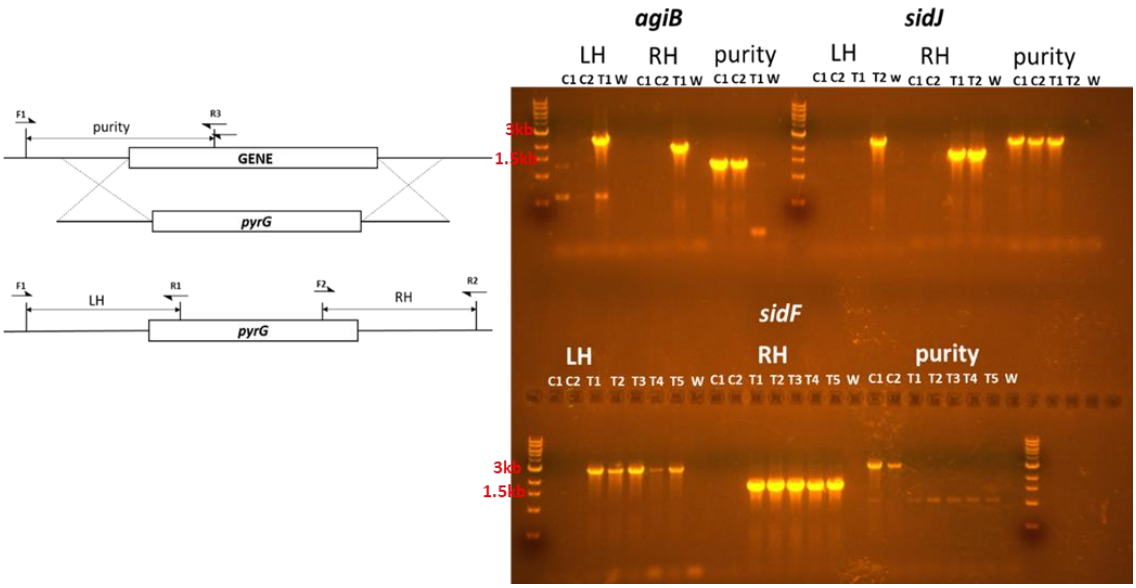


Figure S11. PCR confirmation of the correct disruption of the resistance and purity. Schematic of the expected product (left). Gel for the transfromants C = control; W = water, T = transfromants. Purity test indicates primer that amplify the ORF, and no product should be observed in the correct transfromants. Δ*agiB*; T1 correct LH (2500 bp), RH (1744 bp), and no product in purity test (1286 bp). Δ*sidJ*; T2 correct LH (2426 bp), RH (2005 bp), and no product in purity test (2490 bp). Δ*agiB*; T1, T2, T3, T4, T5 correct LH (2861 bp), RH (1730 bp), and no product in purity test (3034 bp).

Reverse transcriptase quantitative PCR (RT-qPCR)

The wild- type and Δ*sntB* strains were grown in liquid PDB media at 30 °C and 200 rpm. RNA was extracted at day 2, 3 and 6 from lyophilized tissues using Trizol (Invitrogen). For RT-qPCR, RNA was quantified and 10 µg was DNaseI treated before cDNA synthesis using iScript (Bio-Rad). Primers used in qPCR are listed (Table S2). qPCR was performed using iQ SYBR Green Supermix (BIO-RAD) and CFX Connect Real-Time System machine (BIO-RAD). Program used considted of: (1) 95 °C for 3 minutes; (2) 95 °C for 30 seconds; (3) 55 °C for 30 seconds; Steps 2-3 were repeated 39 times.

The relative quantification of gene expression using $2^{-\Delta\Delta C_T}$ (Livak) method. **(1)** The C_T value of the target gene from the Δ*sntB* was normalized to reference gene (actin). **(2)** Then the C_T from WT of the target gene was normalized to reference gene (actin). **(3-4)** The Normalized expression ratio was calculated.

$$(1)\ \Delta C_{T(test)} = C_{T(target,\ test)} - C_{T\,(ref.,\ test)}$$

$$(2)\ \Delta C_{T(calibration)} = C_{T(target,\ calibrator)} - C_{T\,(ref.,\ calibrator)}$$

$$(3)\ \Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$$

$$(4)\ 2^{-\Delta\Delta C_T} = Normalized\ expression\ ratio$$

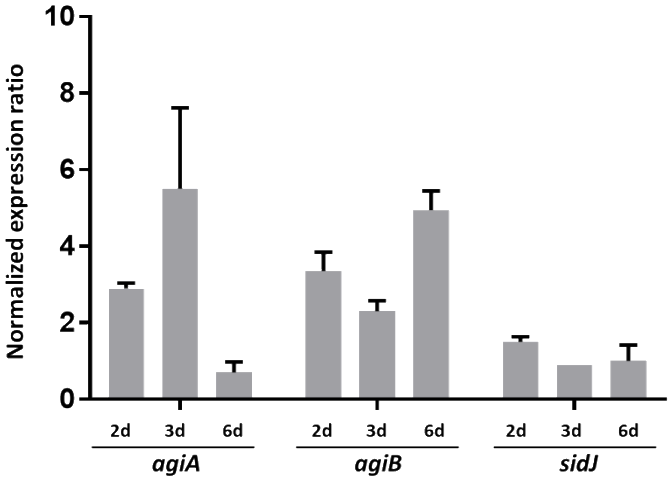


Figure S12. Bar charts showing relative expression of *agiA*, *agiB* and *sidJ* using normalized expression ration ($2^{-\Delta\Delta Ct}$ Method) between WT and Δ*sntB*.

Feeding experiment

The Δ*sntB*/Δ*agiB* strain was inoculated to six 500 mL flasks, each containing 200 mL of PDB. They were grown at 30 °C with shaking at 200 rpm. A solution of *O*-Me-L-tyrosine in water (200 mg/10 mL) was divided over three flasks and added over three days (day 6, 7, 8). Each flask was extracted separately with ethyl acetated. The six extracts (three fed with *O*-Me-L-tyrosine) were analyzed by UPLC-HRMS (Figure S13).

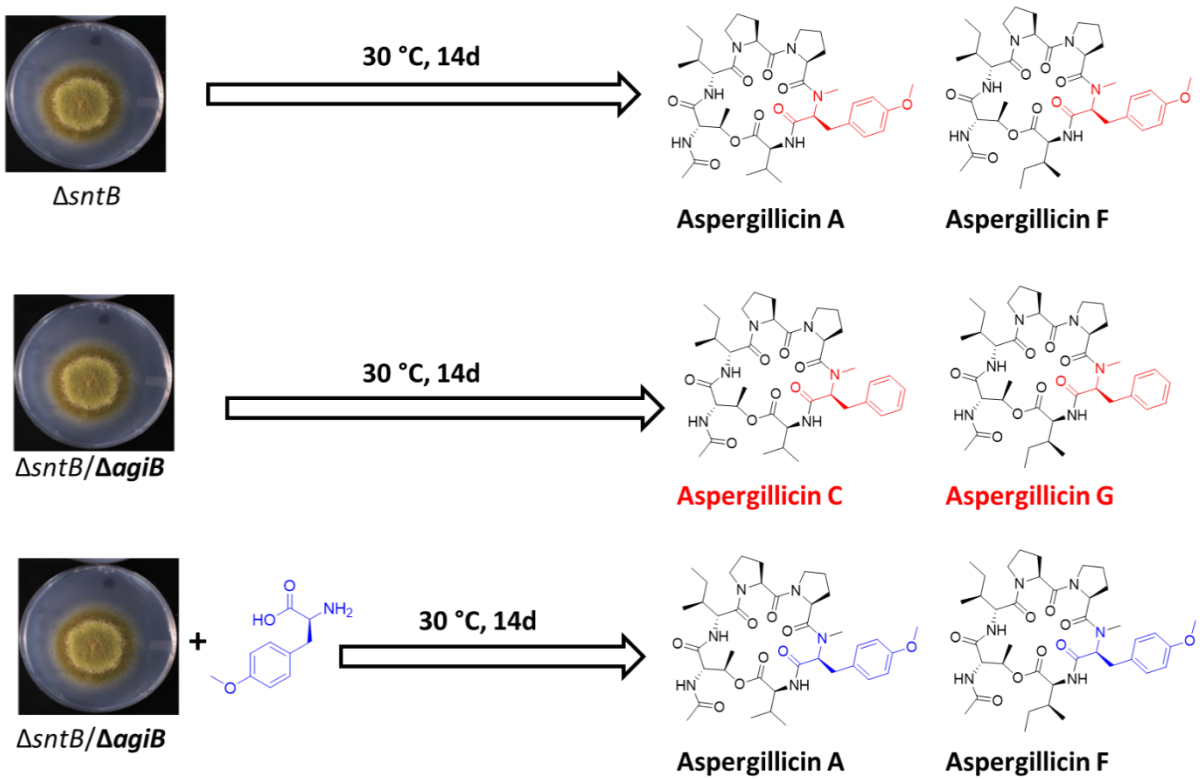


Figure S13. Summary of the feeding experiment using *O*-Me-L-tyrosine. Δ*sntB* produces aspergillicn A **1** and F **2**. Δ*sntB*Δ*agiB* produces aspergillicin C **4** and aspergillicin G **11**. Δ*sntB*Δ*agiB* plus *O*-Me-tyrosine produces aspergillicin A **1** and aspergillicin F **2**.

Sensitivity to iron experiment

The wild type and all the deletion strains were serially passaged for three times on GMM lacking iron and then plated on plates containing either no iron, normal growth media level of $FeSO_4$ (5 µM), high level of $FeSO_4$ (1 mM) and very high level of $FeSO_4$ (10 mM). The plates were grown for 5 days at 30 °C and their growth was evaluated.

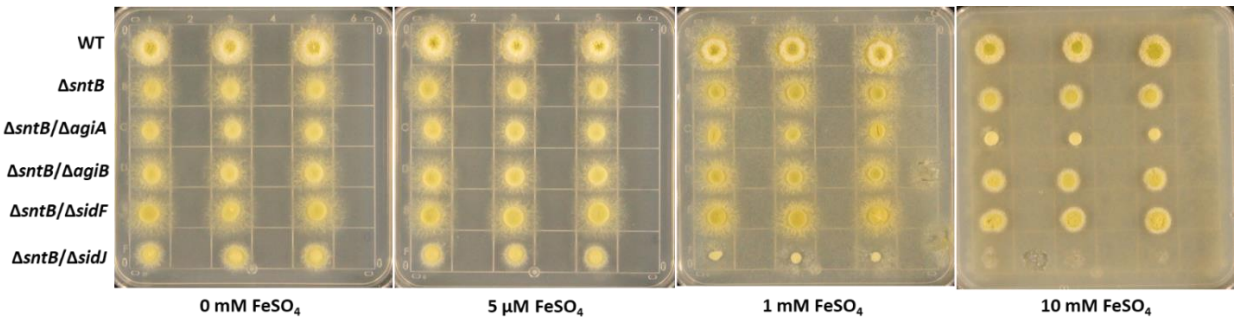
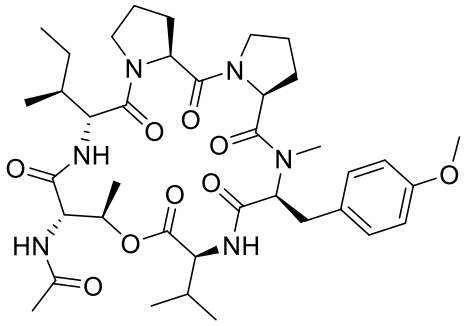


Figure S14. GMM plates contating different amounts of $FeSO_4$ (0, 5 µM, 1 mM and 10 mM) inoculated with WT and Δ*snt*, Δ*sntB*Δ*agiA*, Δ*sntB*Δ*agiB*, Δ*sntB*Δ*sidF* and Δ*sntB*Δ*agiJ*. Picure taken after 5 days.

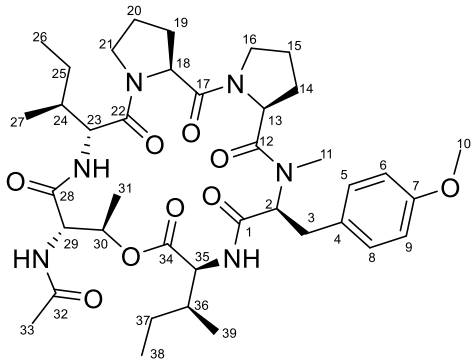
Isolations of aspergillicins

Aspergillicin A 1



A. flavus Δ*sntB* was grown on PDA plates (10 x 25 mL) at 30 °C. After 14 days, it was extracted and purified using prep-HPLC to obtain of pure aspergillicin A **1** as faint yellow powder (1.6 mg). λ_{max} / nm 200, 220, 280; δ_H ($CDCl_3$, 600 MHz) 8.25 (1H, d, 8.5 Hz), 7.10-7.08 (1H, m), 7.04 (2H, d, 8.5 Hz), 6.84 (2H, d, 8.5), 6.62 (1H, d, 9.5 Hz), 5.56 (1H, m), 4.95 (1H, dd, 11.5, 3.3 Hz), 4.86 (1H, dd, 10.0, 2.5 Hz), 4.62 (1H, dd, 9.5, 7.2 Hz), 4.52 (1H, dd, 9.2, 5.2 Hz), 4.42 (1H, dd, 8.5, 5.9 Hz), 4.32 (1H, dd, 8.5, 5.2), 3.78(3H, s), 3.70-3.63 (2H, m), 3.58-3.49 (2H, m), 2.16 (3H, s), 2.20-2.08 (3H, m), 2.0-1.8 (4H, m), 1.74-1.70 (2H, m), 1.39-1.37 (1H, m), **1.30** (3H, m), **1.0-0.85** (13H, m); δ_C ($CDCl_3$, 150 MHz) 173.4, 171.3, 171.25, 170.4, 170.2, 169., 168.6, 130.7 (2C), 130.5, 114.5 (2C), **72.0**, 62.7, 59.0, 58.4, 47.6, 47.5, **47.8**, 38.1, 33.6, 30.5, 29.5, **28.7, 28.0, 26.2, 25.6, 24.8, 23., 19.6, 18.4, 16.7, 14.3, 11.9**; m/z (ESI) 741.4175 $[M+H]^+$ ($C_{38}H_{56}N_6O_9$ requires 741.4187). Data in accordance to the literature.[8] chemical shifts in **bold** were identified using HSQC/HMBC.

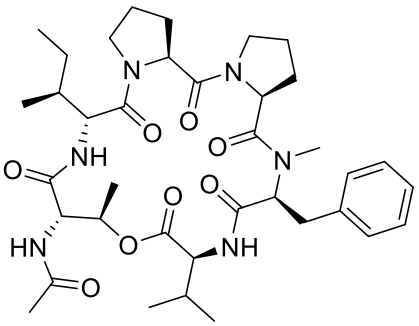
Aspergillicin F 2



A. flavus Δ*sntB* was grown on PDA plates (10 x 25 mL) at 30 °C. After 14 days, it was extracted and purified using prep-HPLC to obtain of pure aspergillicin F **2** as faint yellow powder (8 mg).

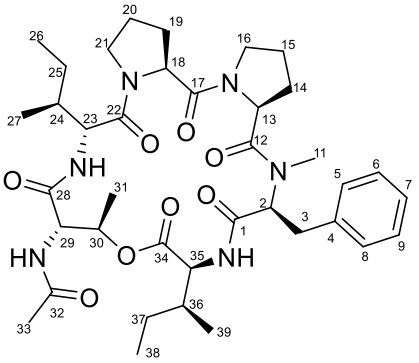
λ_{max} / nm 200, 220, 280; δ_H ($CDCl_3$, 600 MHz) and δ_C ($CDCl_3$, 150 MHz) see Table S4; m/z (ESI) 755.4337 $[M+H]^+$ ($C_{39}H_{58}N_6O_9$ requires 755.4343). Data in accordance to the literature.[8]

Aspergillicin C 4



A. flavus Δ*sntB*Δ*agiB* was grown on PDA plates (10 x 25 mL) at 30 °C. After 14 days, it was extracted and purified using prep-HPLC to obtain of pure aspergillicin C **4** as faint yellow powder (1 mg). λ_{max} / nm 200, 220, 280; δ_H ($CDCl_3$, 600 MHz) 8.26 (1H, d, 8.0 Hz), 7.33-7.26 (3H, m), 7.21-7.10 (3H, m), 6.63 (1H, d, 9.7 Hz), 5.60-5.54 (1H, m), 5.01 (1H, dd, 11.8, 3.6), 4.87,(1H, dd, 10.0, 2.4 Hz), 4.63 (1H, dd, 9.6, 7.5 Hz), 4.51 (1H, m), 4.41 (1H, dd, 8.6, 6.2 Hz), 4.27 (1H, m), 3.70-3.47 (4H, m), 3.22, (1H, dd, 14.2, 3.0 Hz), 3.04 (1H, dd, 14.2, 11.5), 2.85 (3H, s), 2.17 (3H, s), 2.25-2.18 (2H, m), 2.01-1.84 (4H, m), 1.70-1.62 (2H, m), 1.43-1.38 (1H, m), 1.27 (3H, d, 15 Hz), 1.16-1.09 (1H, m), 1.8-0.75 (14H, m); m/z (ESI) 711.4072 $[M+H]^+$ ($C_{37}H_{54}N_6O_8$ requires 711.4081). Data in accordance to the literature.[9]

Aspergillicin G 11



A. flavus Δ*sntB*Δ*agiB* was grown on PDA plates (10 x 25 mL) at 30 °C. After 14 days, it was extracted and purified using prep-HPLC to obtain of pure aspergillicin G **11** as faint yellow powder (5 mg). λ_{max} / nm 200, 220, 280; ^{1}H NMR and ^{13}C NMR data see Table S5; m/z (ESI) 725.4232 $[M+H]^+$ ($C_{38}H_{56}N_6O_8$ requires 725.4237).

HRMS and UV spectra

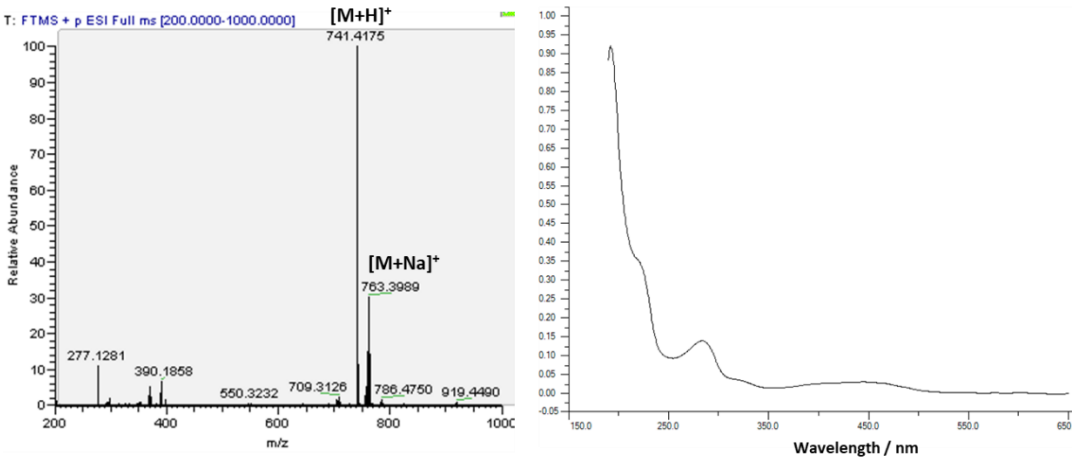


Figure S15. HRMS spectrum (left) and UV spectrum of aspergillicin A **1**.

Aspergillicin F

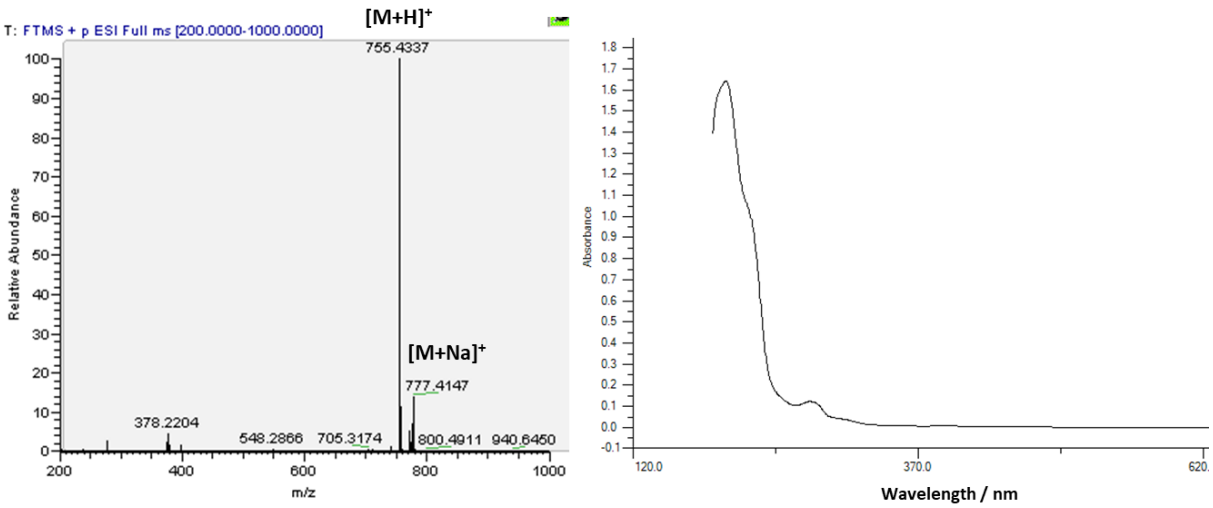


Figure S16. HRMS spectrum (left) and UV spectrum of aspergillicin F **2**.

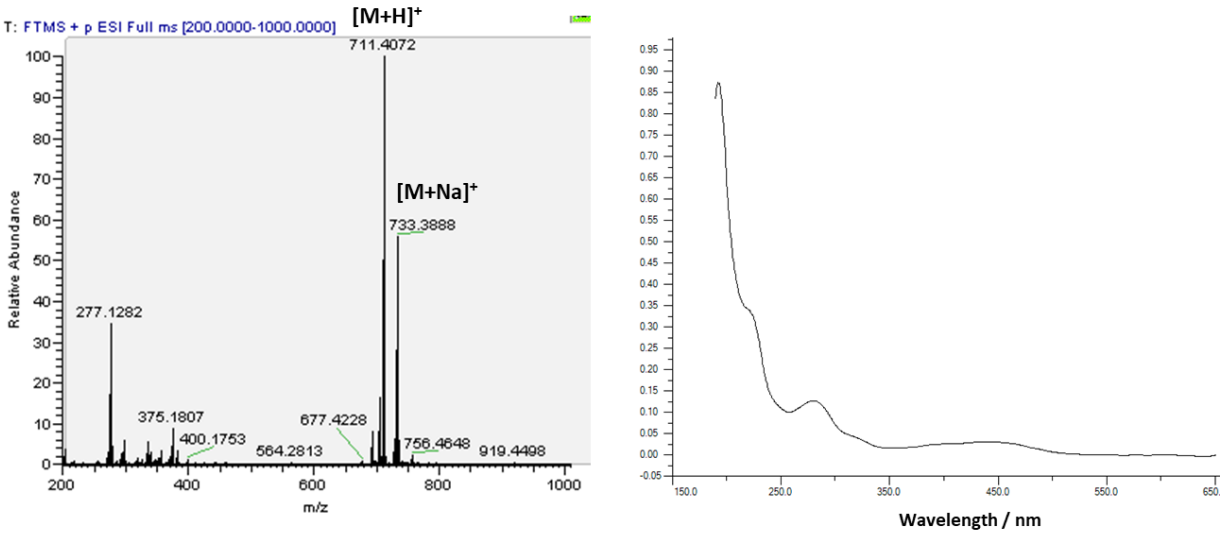


Figure S17. HRMS spectrum (left) and UV spectrum of aspergillicin B **4**.

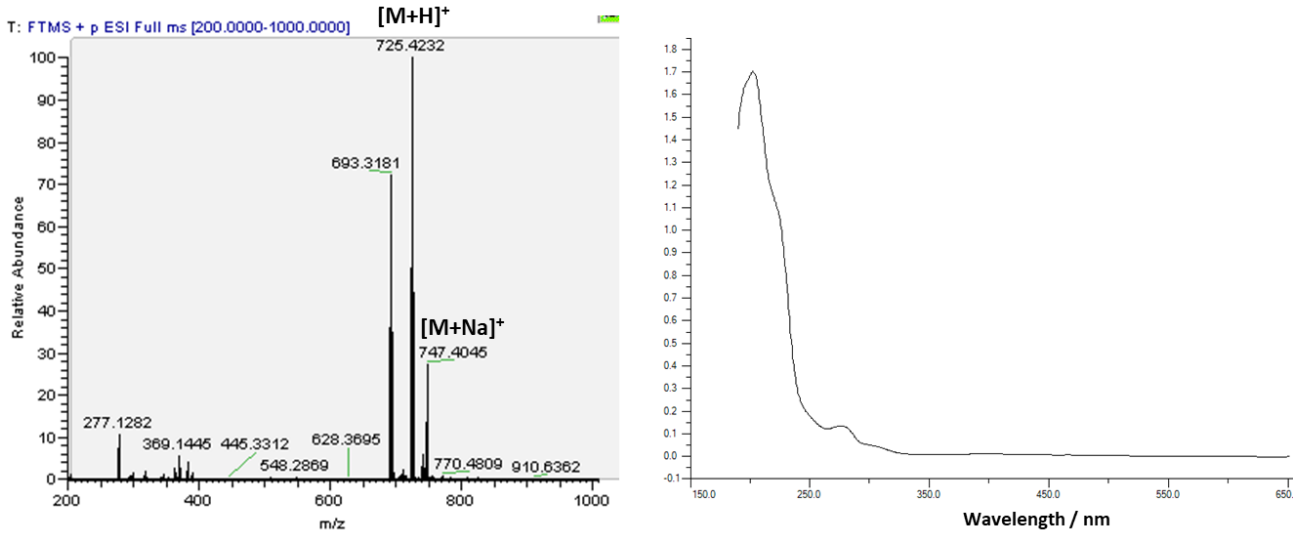


Figure S18. HRMS spectrum (left) and UV spectrum of aspergillicin G **11**.

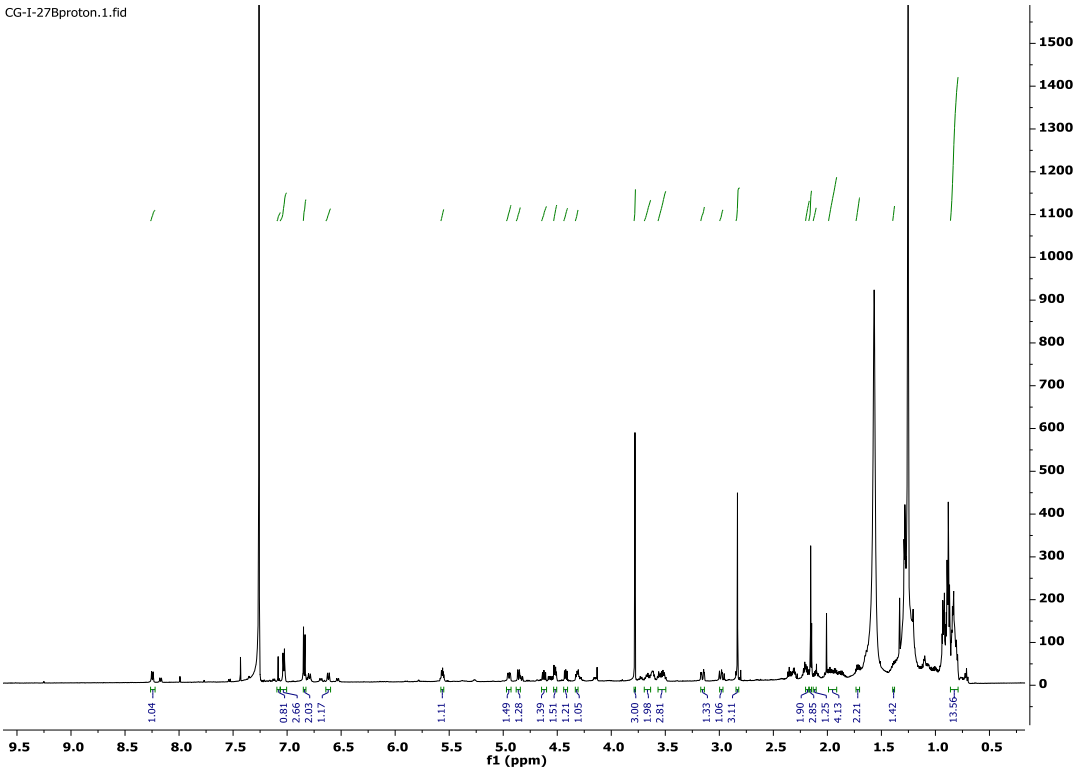
NMR data

Table S4. NMR assignment of aspergillicin F **2** in agreement with literature[8] ([a]150 MHz, [b]600 MHz in $CDCl_3$).

Aspergillicin F			
Residue	**Position**	**δ_C[a] / ppm**	**δ_H[b] / ppm (*J* in Hz)**
***N*-Me-L-*O*-Me-Tyr**	1	169.8	-
	2	62.7	4.95 (1H, dd, 11.5, 3.4)
	3a	33.7	3.17 (1H, dd,14.7, 3.4)
	3b		2.98 (1H, dd, 14.7, 11.5)
	4	129.8	-
	5/9	130.6	7.03 (2H, d, 8.6)
	6/8	114.5	6.84 (2H, d. 8.6)
	7	158.9	-
	10	55.6	3.78 (3H, s)
	11	29.5	2.83 (3H, s)
L-Pro	12	173.4	-
	13	55.3	4.31 (1H, dd, 8.0, 5.0)
	14a	28.7	1.07 (1H, m)
	14b		0.87 (1H, m)
	15a	25.5	2.11 (1H, m)
	15b		1.71 (1H, m)
	16a	47.6	3.62 (1H, m)
	16b		3.52 (1H, m)
L-Pro	17	170.2	-
	18	58.3	4.51 (1H, dd, 8.5, 5.2)
	19a	28.1	2.20 (1H, m)
	19b		1.84 (1H, m)
	20a	24.8	1.98 (1H, m)
	20b		1.93 (1H, m)
	21a	47.8	3.7 (1H, m)
	21b		3.6 (1H, m)
D-*allo*-Ile	22	171.3	-
	23	54.9	4.61 (1H, dd, 9.9, 8.0)
	23-NH	-	6.54 (1H, d, 9.90)
	24	37.8	1.67 (1H, m)
	25	26.2	1.38 (1H, m)
			1.07 (1H, m)
	26	11.9	0.91 (3H, m)
	27	14.7	0.88 (3H, m)
***O*-Ac-L-Thr**	28	168.8	-
	29	55.9	4.85 (1H, dd, 9.8, 2.2)
	29-NH	-	7.03 (1H, m)
	30	71.6	5.56 (1H, dq, 6.6, 2.2)
	31	16.8	1.28 (3H, d, 6.6)
	32	171.25	-
	33	23.3	2.14 (3H, s)
L-Ile	34	170.4	-
	35	57.7	4.55 (1H, dd, 8.8, 5.6)
	35-NH	-	8.17 (1H, d, 8.8)
	36	37.0	1.95 (1H, m)
	37	24.8	1.38 (1H, m)
			1.14 (1H, m)
	38	11.3	0.83 (3H, t, 7.4)
	39	15.8	0.90 (3H, m)

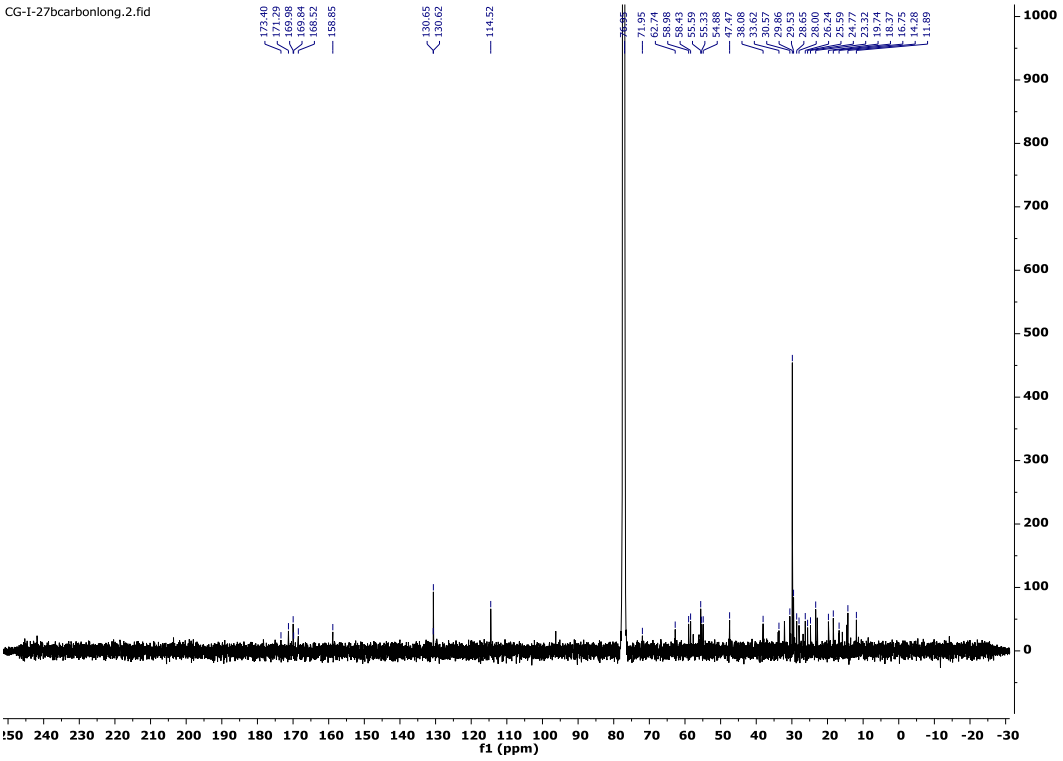
Table S5. NMR assignment of aspergillicin G **11** ([a]150 MHz, [b]600 MHz in $CDCl_3$).

Aspergillicin G					
Residue	**Position**	**δ_C[a] / ppm**	**δ_H[b] / ppm (*J* in Hz)**	**HMBC**	**COSY**
***N*-Me-L-Phe**	1	169.9	-	-	-
	2	62.7	5.00 (1H, dd, 11.5, 3.3)	C1	3a/3b
	3a	34.6	3.23 (1H, dd,14.5, 3.3)	C1, C4, C5/9	2, 3b
	3b		3.03 (1H, dd, 14.5, 11.5)		2, 3a
	4	138.0	-	-	-
	5/9	129.1	7.13 (2H, m)	C3, C7	6/8, 7
	6/8	129.7	7.31 (2H, m	C7, C5/9	5/9, 7
	7	127.2	7.25 (1H, m)	C5/9	6//8, 5/9
	11	29.9	2.85 (3H, s)	C12	-
L-Pro	12	173.4	-	-	-
	13	55.3	4.27 (1H, dd, 8.0, 5.0)	C14, C15	14a/b
	14a	28.6	0.97 (1H, m)	C12, C13,	13, 14b, 15a, 15b
	14b		0.71 (1H, m)		13, 14a, 15a, 15b
	15a	25.5	2.11 (1H, m)	C14, C13	15a, 15b, 16a, 16b
	15b		1.71 (1H, m)		15a, 15b, 16a, 16b
	16a	47.6	3.62 (1H, m)	C14, C13,	15a,15b, 16b
	16b		3.52 (1H, m)		15a, 15b, 16a
L-Pro	17	170.2	-	-	
	18	58.3	4.50 (1H, dd, 8.5, 5.2)	C17, C19, C20	19a/19b
	19a	28.0	2.20 (1H, m)	C17, C20, C21	18,19b
	19b		1.84 (1H, m)		
	20a	24.8	1.98 (1H, m)	C19, C21	18, 19a
	20b		1.93 (1H, m)		
	21a	47.8	3.69 (1H, m)	C19, C20	20a, 20b, 21b
	21b		3.62(1H, m)		20a, 20b, 21a
D-*allo*-Ile	22	171.3	-	-	-
	23	55.0	4.62 (1H, dd, 9.9, 8.0)	C22, C24, C25, C27, C28	23NH, 24
	23-NH	-	6.56 (1H, d, 9.90)	C28	23
	24	37.8	1.67 (1H, m)	C23	23, 25, 27
	25	26.2	1.38 (1H, m)	C24, C26, 27	24, 26, 27
			1.07 (1H, m)		
	26	11.8	0.91 (3H, m)	C23, C24, C25	25
	27	14.7	0.88 (3H, m)	C23, C24, C25	25
***O*-Ac-L-Thr**	28	168.7	-	-	-
	29	55.9	4.83 (1H, dd, 9.8, 2.2)	C28	29-NH, 30
	29-NH	-	6.8 (1H, d, 9.8)	-	29
	30	71.7	5.57 (1H, qd, 6.4, 2.2)	C34	29, 31
	31	16.9	1.28 (3H, d, 6.4)	C29, C30	29
	32	171.12	-	-	-
	33	23.3	2.14 (3H, s)	C32	-
L-Ile	34	170.2	-	-	-
	35	57.8	4.56 (1H, dd, 8.6, 5.6)	C34, C36, C39	35-NH, 36
	35-NH	-	8.18 (1H, d, 8.6)	C34	35
	36	37.1	1.95 (1H, m)	-	35, 37a, 37b, 39
	37	24.89	1.38 (1H, m)	-	36, 37a, 38
			1.14 (1H, m)		36, 37b, 38
	38	11.3	0.83 (3H, t, 7.4)	C36, C38, C39C37,	37a, 37b
	39	15.8	0.90 (3H, m)	-	38



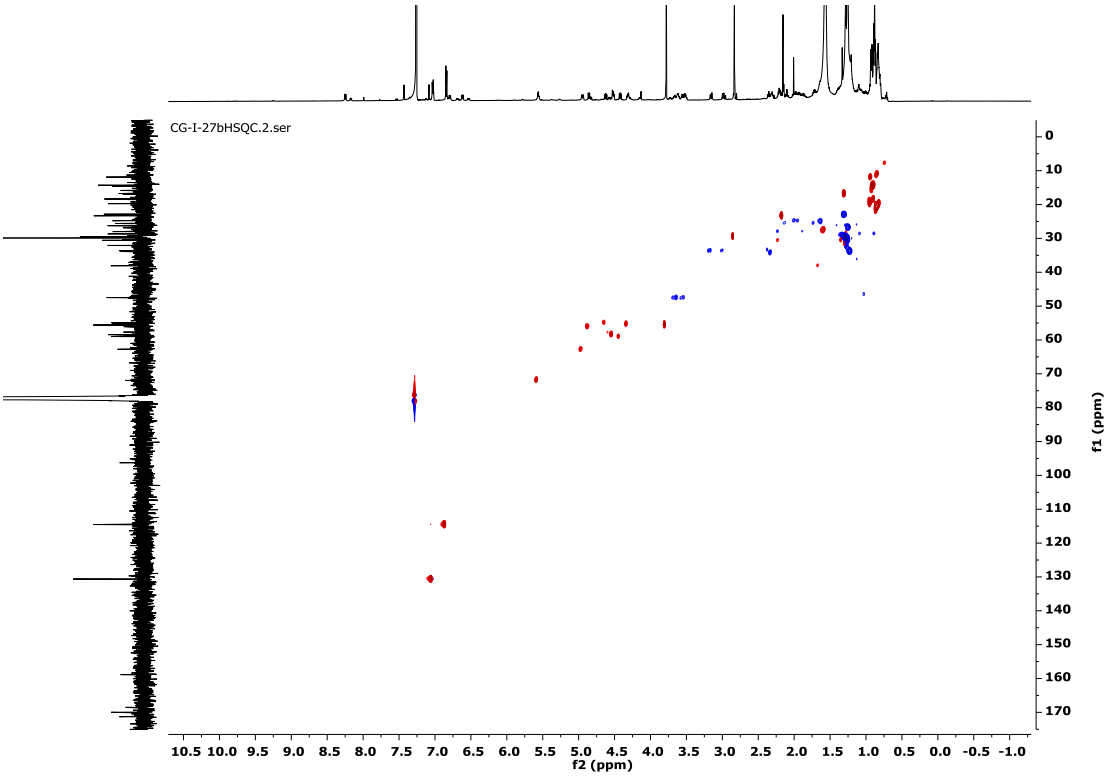
331

332 **Figure S19.** ^{1}H NMR of aspergillicin A **1** (600 MHz in $CDCl_3$).



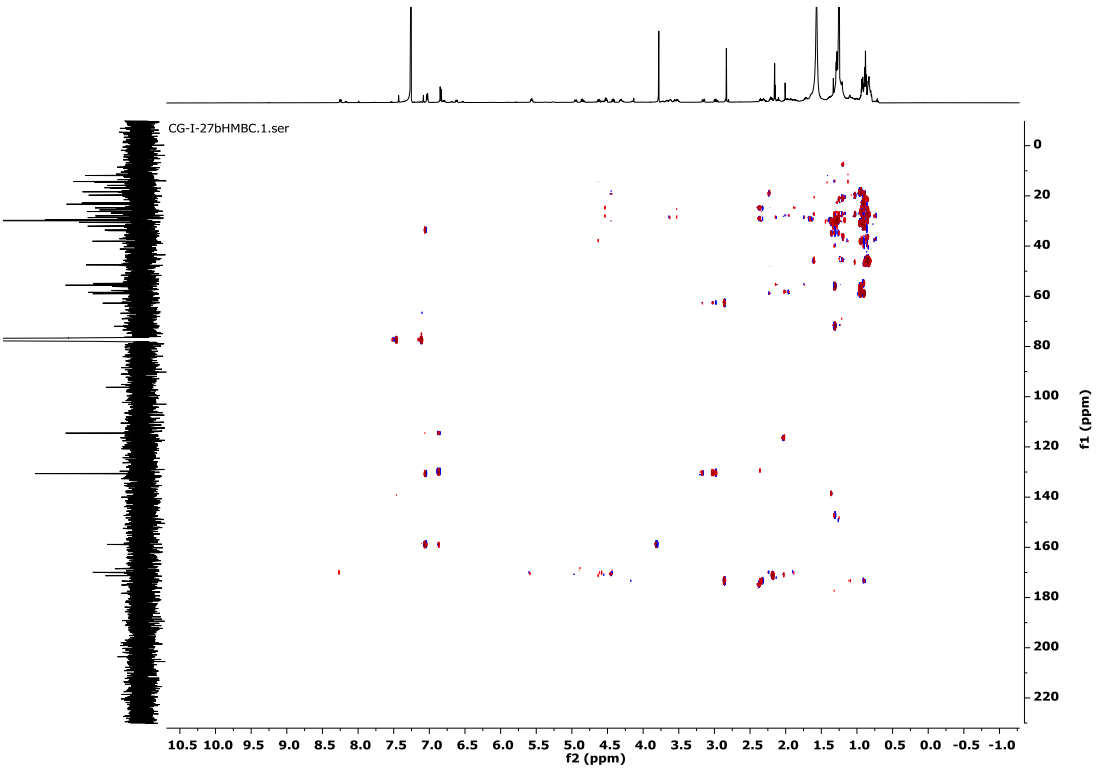
333

334 **Figure S20.** ^{13}C NMR of aspergillicin F **1** (150 MHz in $CDCl_3$)



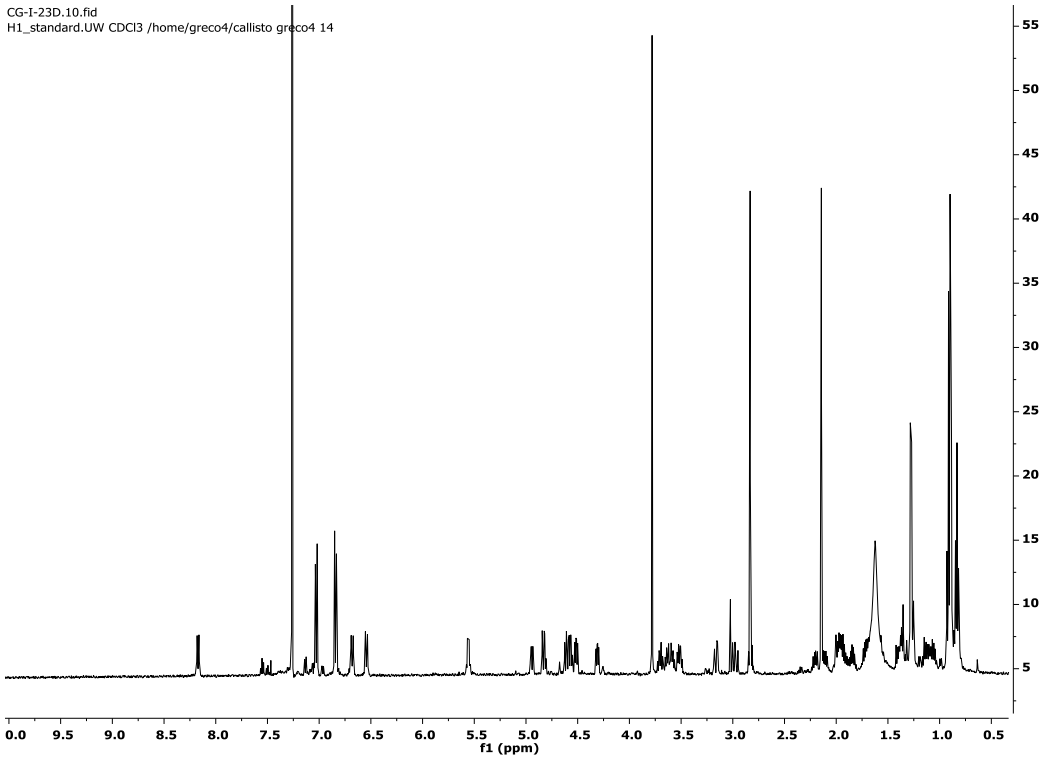
335

336 **Figure S21.** HSQC spectrum of aspergillicin A **1** (600 MHz in $CDCl_3$).



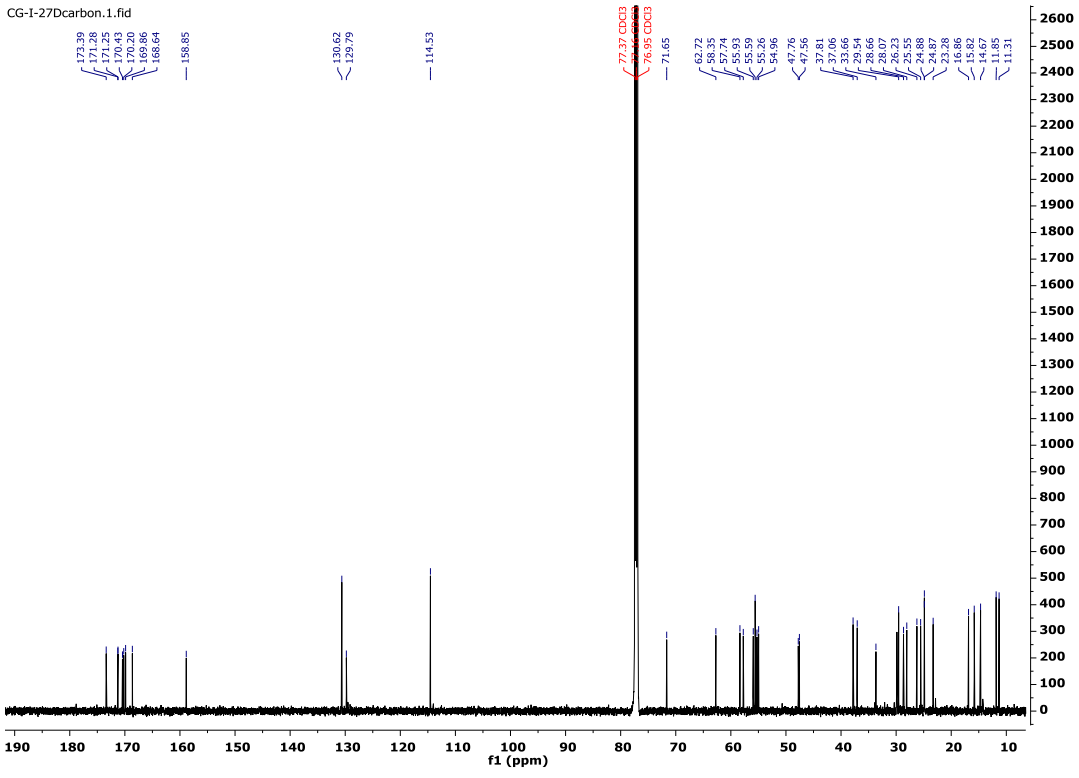
337

338 **Figure S22.** HMBC spectrum of aspergillicin A **1** (600 MHz in $CDCl_3$).



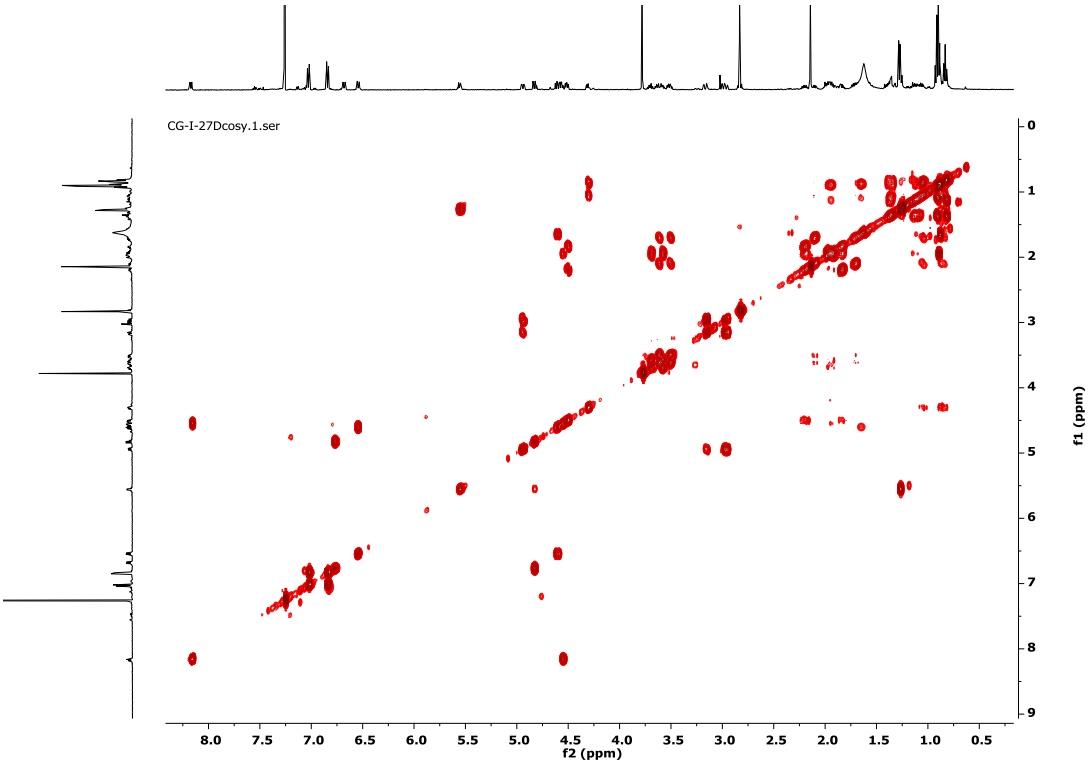
339

340 **Figure S23.** ^{1}H NMR of aspergillicin F **1** (600 MHz in $CDCl_3$).



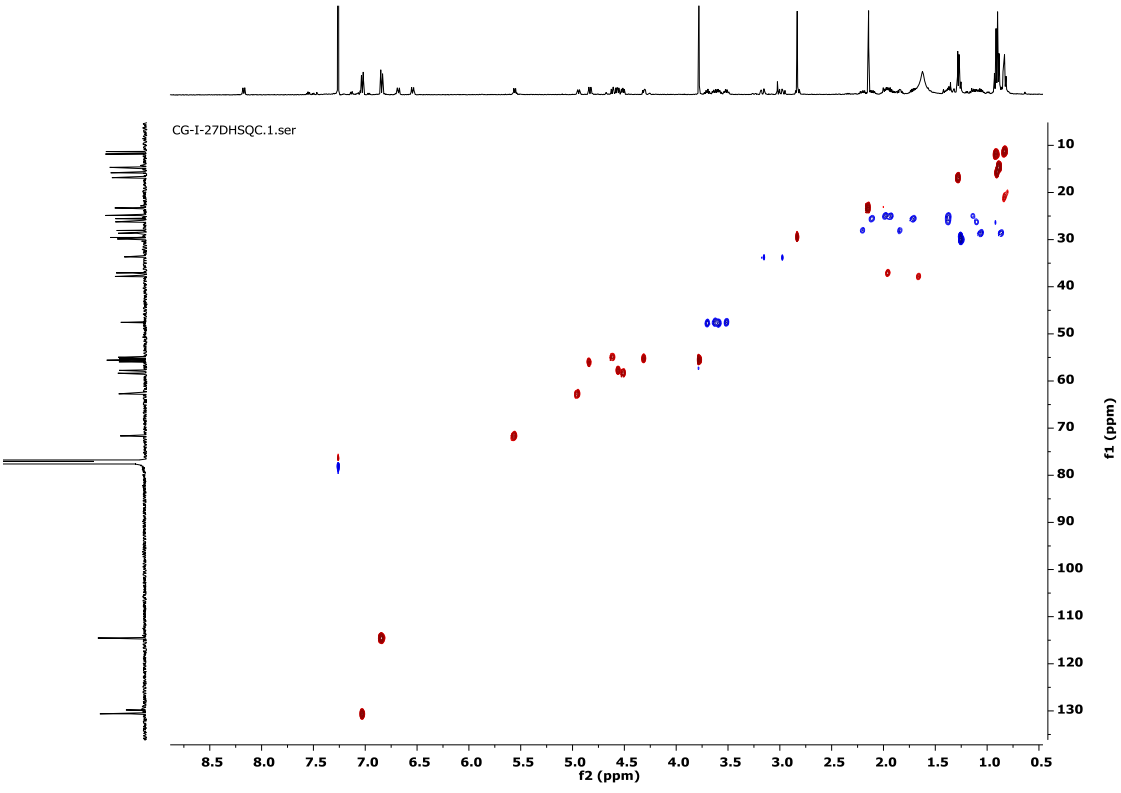
341

342 **Figure S24.** ^{13}C NMR of aspergillicin F **2** (150 MHz in $CDCl_3$)



343

344 **Figure S25.** COSY spectrum of aspergillicin F **2** (600 MHz in $CDCl_3$).



345

346 **Figure S26.** HSQC spectrum of aspergillicin F **2** (600 MHz in $CDCl_3$)

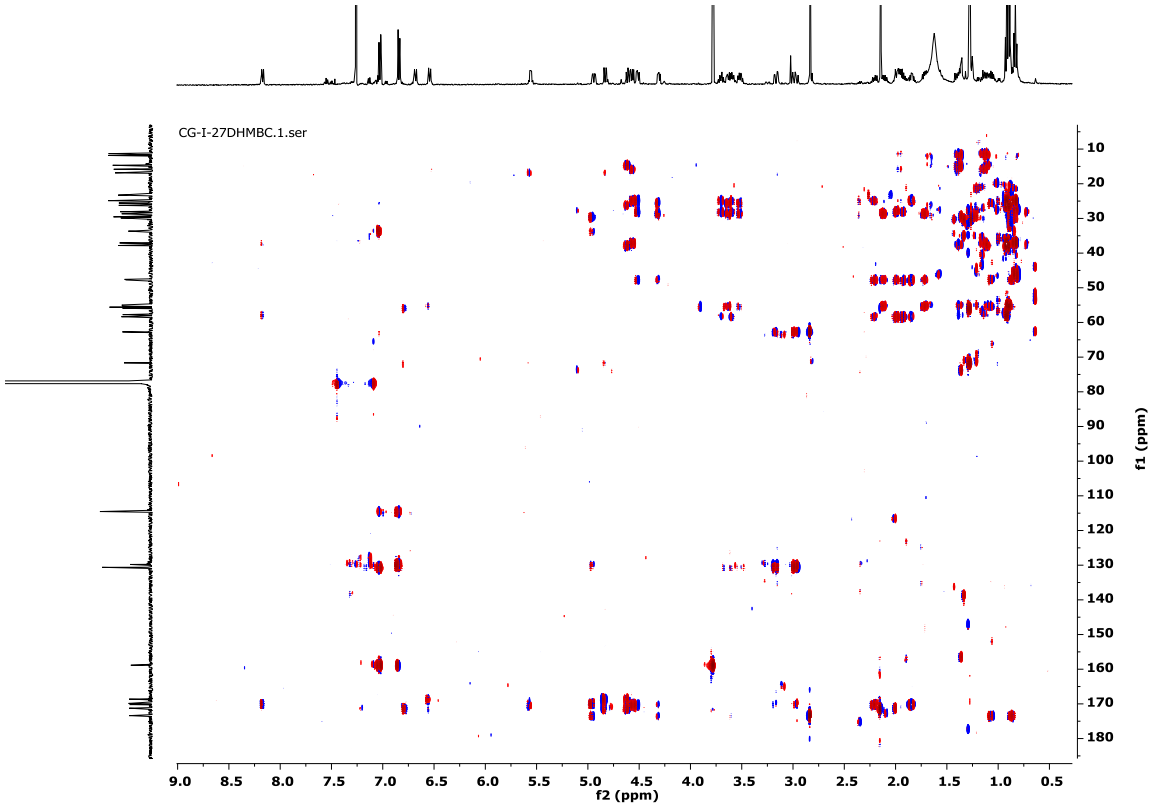


Figure S27. HMBC spectrum of aspergillicin F **2** (600 MHz in $CDCl_3$).

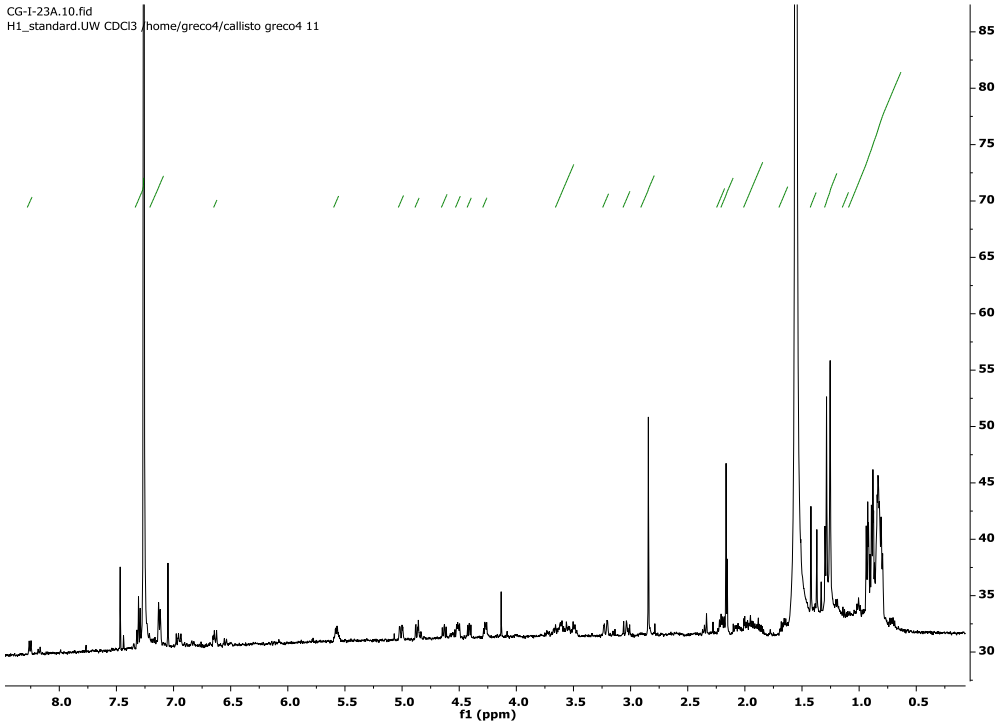


Figure S28. ^1H NMR of aspergillicin C **4** (600 MHz in $CDCl_3$).

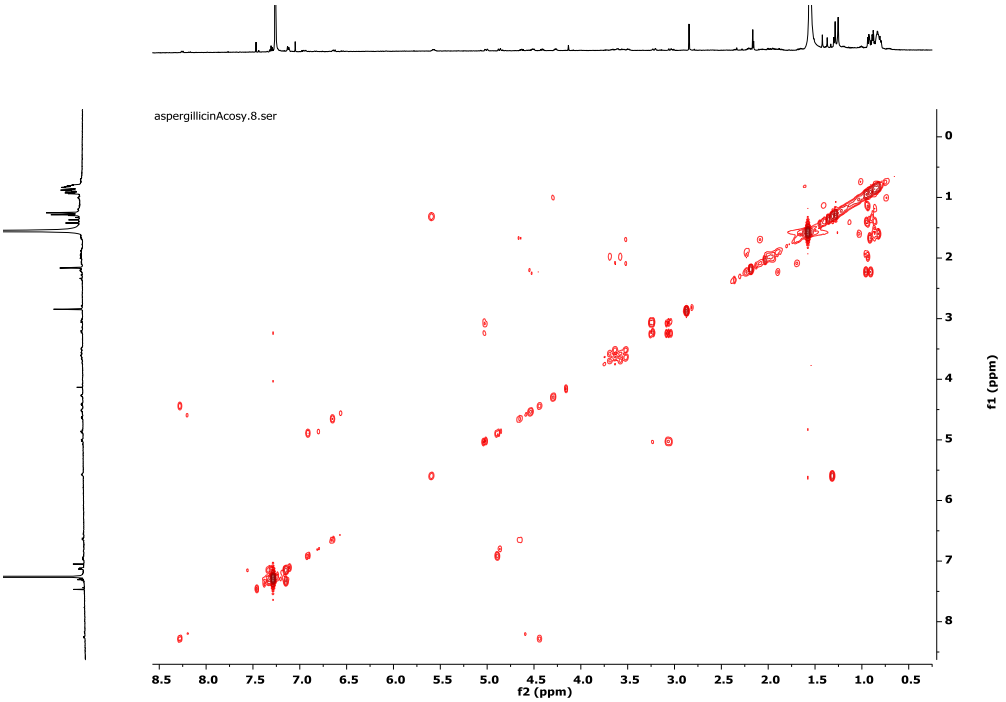


Figure S29. COSY spectrum of aspergillicin C **4** (600 MHz in $CDCl_3$).

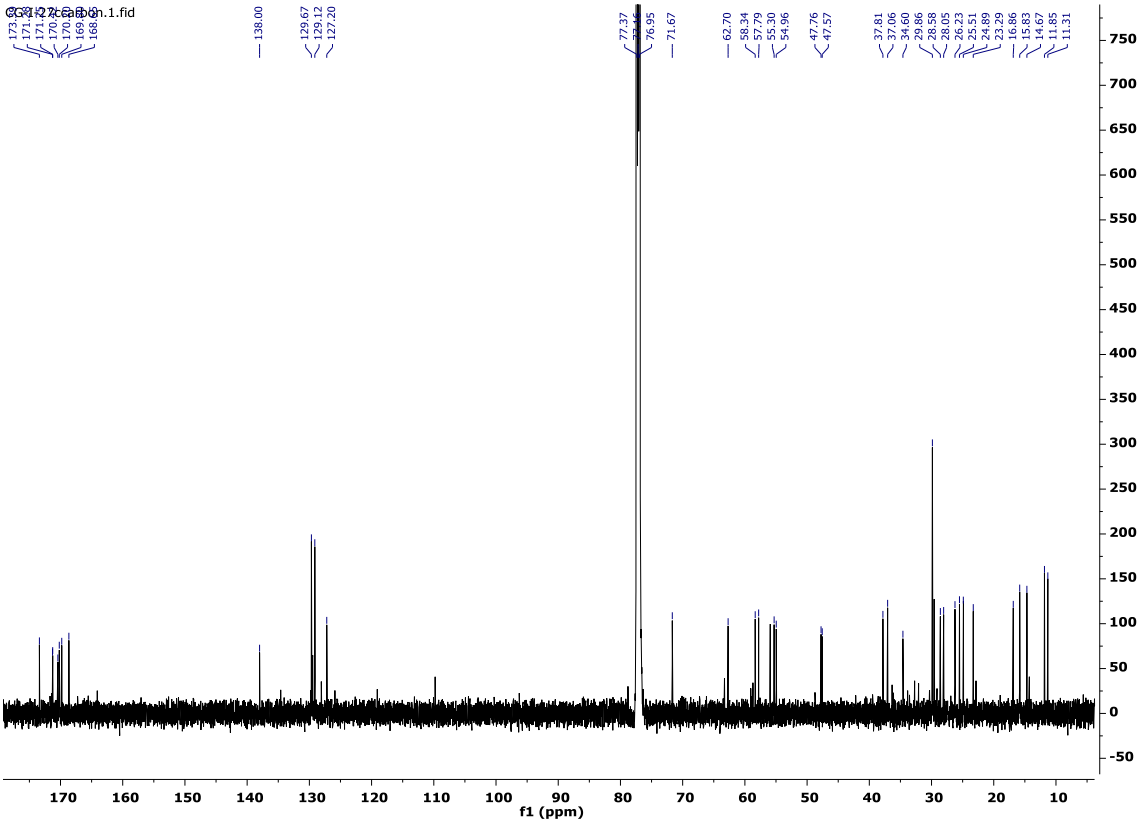
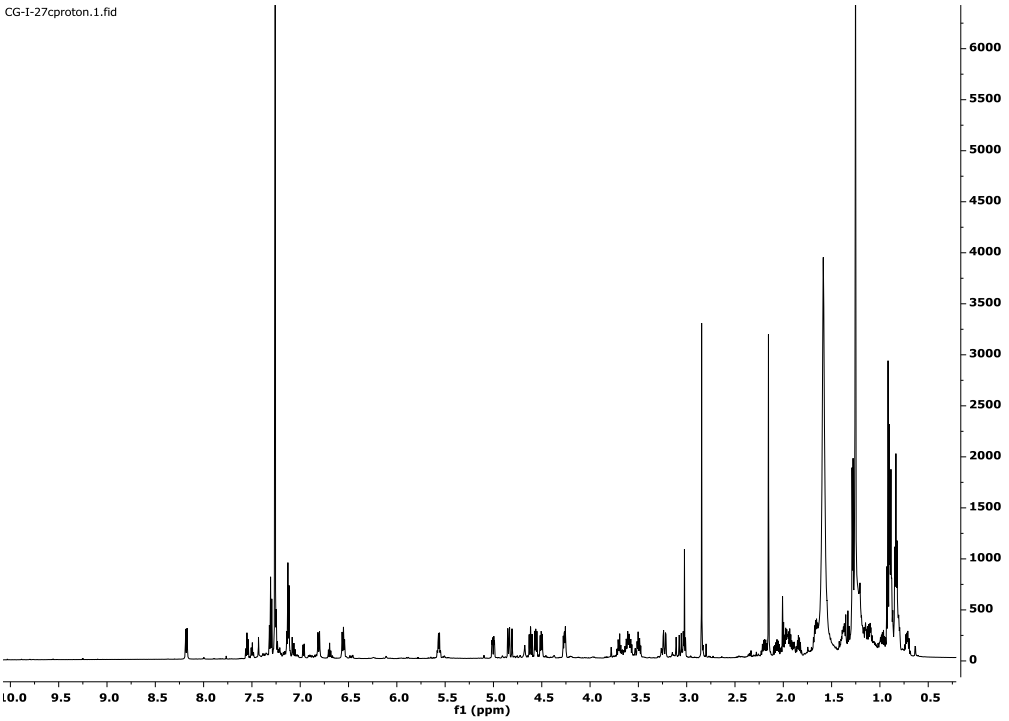
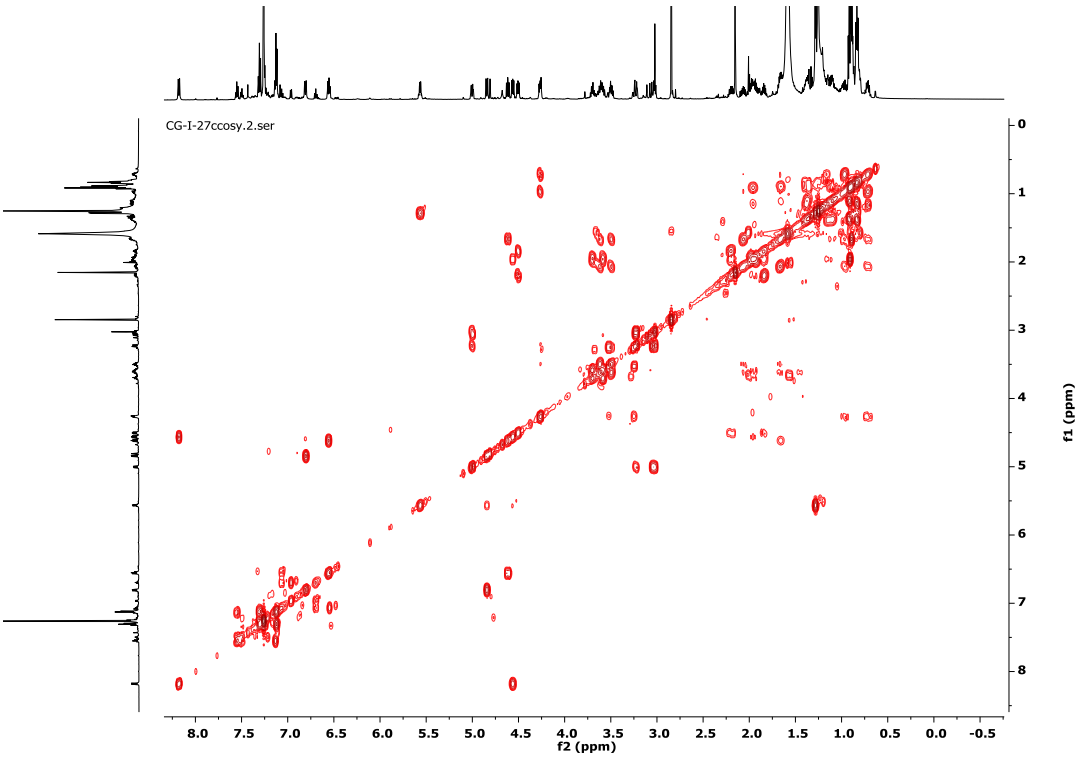


Figure S30. ^{13}C NMR of aspergillicin G **11** (150 MHz in $CDCl_3$).



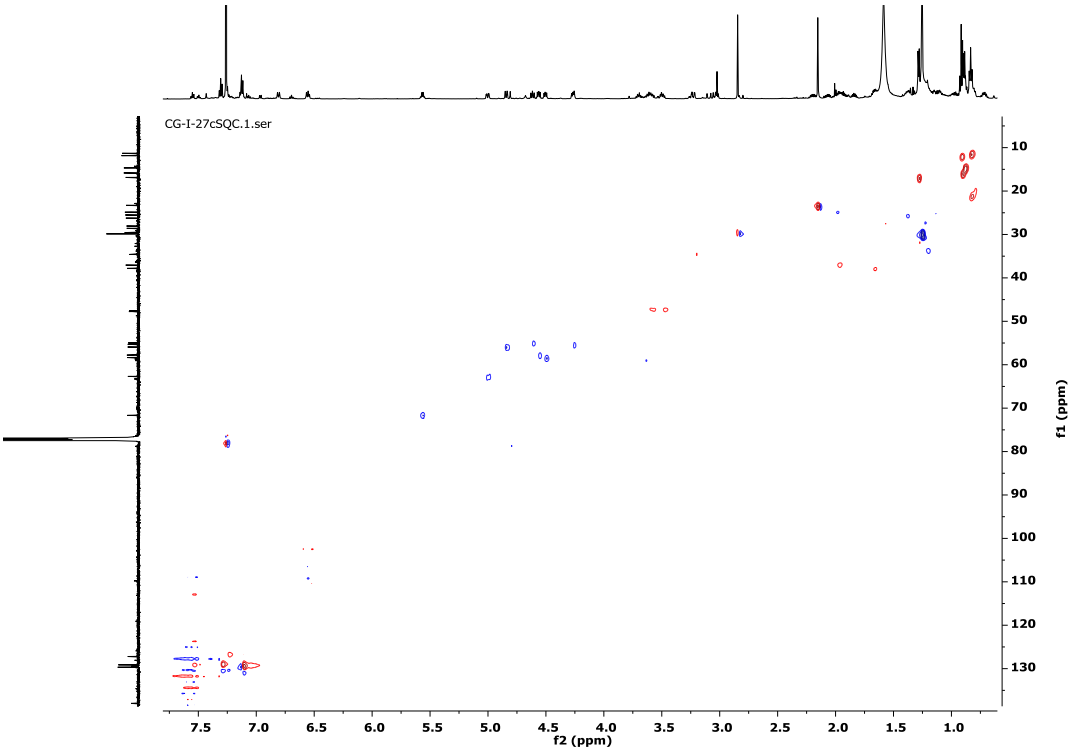
355

356 **Figure S31.** ^{1}H NMR of aspergillicin G **11** (600 MHz in $CDCl_3$).



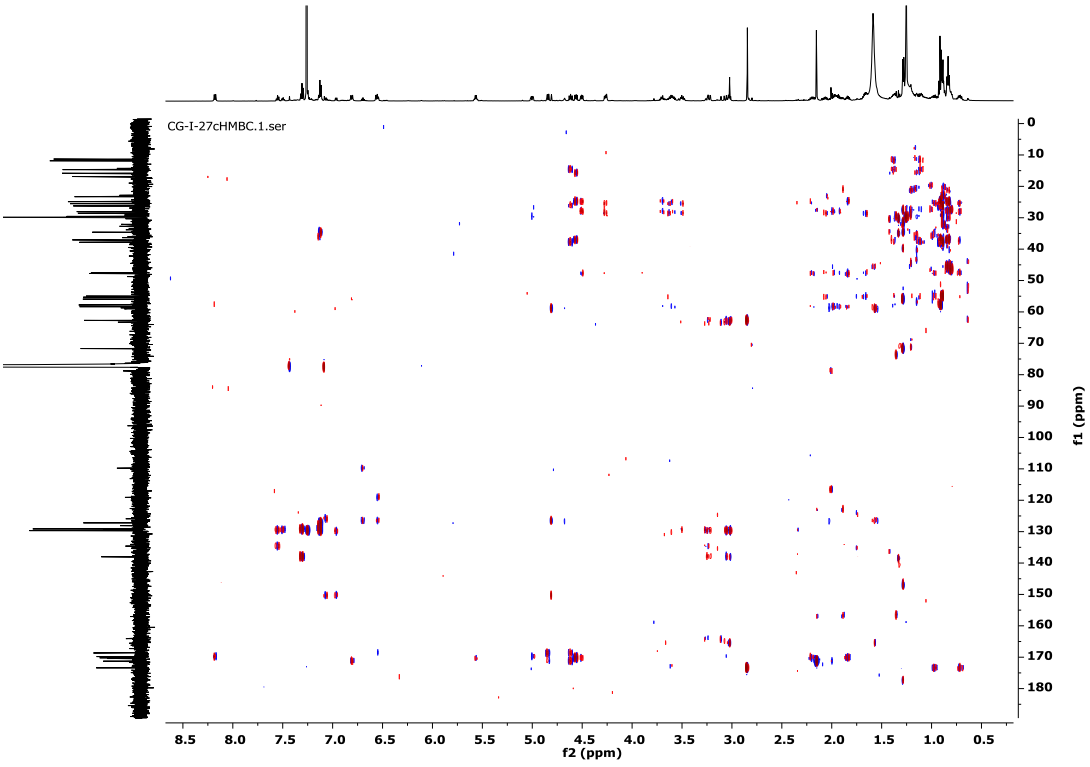
357

358 **Figure S32.** COSY spectrum of aspergillicin G **11** (600 MHz in $CDCl_3$).



359

360 **Figure S33.** HSQC spectrum of aspergillicin G **11** (600 MHz in $CDCl_3$).



361

362 **Figure S34.** HMBC spectrum of aspergillicin G **11** (600 MHz in $CDCl_3$).

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