Supplementary Information

Unexpected assembly machinery for 4(3*H*)quinazolinone scaffold synthesis

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1. Supplementary Methods

1.1 Strains and culture conditions

Fusarium tricinctum CGMCC 3.4731 was cultured at 25 °C, 7 days on PDA medium (26 g/L Potato Dextrose Water, 2% agar) for sporulation or culture at 25 °C on PDB medium for 4 days to extract genome DNA (gDNA). *A. nidulans* L08030 was culture at 37 °C for 3-4 days on solid CD medium (10 g/L glucose, 50 mL/L 20 × nitrate salts, 1 mL/L trace elements, 20 g/L agar) containing 10 mM uridine, 5 mM uracil, 1 µg/mL pyridoxine HCl and 0.25 µg/mL riboflavin for sporulation. The *A. nidulans* mutants was culture at 25 °C for 3.5 days on solid CD-ST medium (20 g/L starch, 10 g/L casein hydrolysate (acid), 50 mL/L nitrate salts, 1 mL/L trace elements, 20 g/L Agar) for heterologous expression and compounds production.

1.2 Isolation of the gDNA and cDNA synthesis

Fusarium tricinctum was cultured in PDB medium (26 g/L Potato Dextrose Water) at 25 °C and 220 rpm for 4 days. The mycelium was collected and used for gDNA extraction by Cetyltrimethylammonium Bromide (CTAB) method (20 g/L CTAB, 81.8 g/L NaCl, 186.1 g /L Na2EDTA·2H₂O and 0.1 M Tris-HCl pH 8.0). The total RNA of the *A. nidulans::ftchy* was isolated from the strain grown in liquid CD-ST production medium (20 g/L starch, 20 g/L casein hydrolysate, 50 mL/L nitrate salts and 1 mL/L trace elements) at 25 °C and 220 rpm for 3 days. RNA was extracted by TRLZOL[®] Reagent (Ambion), followed by cDNA reverse transcription with the Transcriptor First Strand cDNA Synthesis Kit (Roche).

1.3 Plasmid construction

The sequence of *ftchy* gene cluster is deposited in GenBank under accession number OP651004. The gDNA sequences of *ftchy* gene, the protein sequences of *ftChy* protein, the primer sequences are listed in the Source Data file. The plasmids are summarized in **Supplementary Table 1**.

To construct the expression plasmids of *ftchyA~H* genes for *A. nidulans*, each gene with its terminator (~ 400 bp) was amplified from the gDNA of *F. tricinctum* CGMCC 3.4731 by PCR using primer pairs pANR-*ftchyA*-F1/R1, pANR-*ftchyA*-F2/R2, pANR-*ftchyA*-F3/R3, pANU-*ftchyC*-F/R, pANU-*ftchyD*-F/R, pANU-*ftchyE*-F/R, pANP-*ftchyM*-F/R, pANP-*ftchyH*-F/R, pANU-*ftchyC*-F/R2, pANU-*ftchyH*-F/R2, pANP-*ftchyM*-F2/R yielding plasmids pIM 3201-3206, through yeast homologous recombination in *S. cerevisiae* BJ5464-NpgA. The *glaA*, *gpdA* and *amyB* promoters were amplified from vectors pANU, pANR and pANP by using primer pairs *glaA*-F/*glaA*-R, *gpdA*-F/*gpdA*-R and *amyB*-F/*amyB*-R, respectively. Gene *ftchyA* was cloned into vector pANR with *gpdA* promoter yielding plasmid pIM 3201. Genes *ftchyC*, *ftchyD* and *ftchyE* were cloned into vector pANU yielding plasmid pIM 3202, while the promoters *gpdA*, *glaA* and *amyB* were used for *ftchyC*, *ftchyD* and *ftchyE*, respectively.

Genes *ftchyM* and *ftchyH* were cloned into vector pANP yielding plasmid pIM 3203, while the promoters *gpdA* and *amyB* were used for *ftchyM* and *ftchyH*, respectively. Genes *ftchyC* and *ftchyD* were cloned into vector pANU yielding plasmid pIM 3204, while the promoters *gpdA* and *glaA* were used for *ftchyC* and *ftchyD*, respectively. Genes *ftchyH* and *ftchyM* were cloned into vector pANP with the promoter *amyB* yielding plasmid pIM 3205 and pIM 3206, respectively.

To express *ftchyA* in *E. coli* BL21, intron-free *ftchyA* was cloned from the cDNA of A. nidulans containing ftchy cluster using the primer pairs pYEU-ftchyA-F1/R1, pYEUftchyA-F2/pANR-ftchyA-R1, pANR-ftchyA-F2/R2, pANR-ftchyA-F3/pYEU-ftchyA-R2. The corresponding overlapping fragments were ligated to vector pYEU by yeast homologous recombination to obtain plasmid pIM 3207. The plasmid pIM 3207 was further digested with BamH I and EcoR I to obtain ftchyA and subsequently inserted into the BamH I and EcoR I digested pColdI (an expression vector with His tag on Nterminus) to yield plasmid pIM 3208. The mutated expression plasmids pIM 3209-3211 were constructed through yeast homologous recombination in S. cerevisiae BJ5464-NpgA. The mutated fragments were amplified by PCR using primer pairs pYEUftchyA-F1/pANR-ftchyA-R1, pANR-ftchyA-F2/pYEU-ftchyA-C1-H987A-R, pYEUftchyA-C₁-H₉₈₇A-F/pANR-ftchyA-R2, pANR-ftchyA-F3/pYEU-ftchyA-R2, pANRftchyA-F2/pYEU-ftchyA-C₁*-R, pYEU-ftchyA-C₁*-F/pANR-ftchyA-R2, pANR-ftchyA-F3/pYEU-*ftchyA*- C_T - $H_{2075}A$ -R, pYEU-*ftchyA*- C_T - $H_{2075}A$ -F/pYEU-*ftchyA*-R2. The plasmids pIM 3209-3211 were digested and ligated in to pColdI by same method to generate plasmids pIM 3212-3214, respectively. To express the stand-alone ftChyA-A₁, ftChyA-A₂ and ftChyA-C_T domain in *E. coli* BL21, the plasmids pIM 3215-3217 were constructed, respectively. The intron-free *ftchyA-A*₁ was amplified by PCR using primer pairs pColdI-ftchyA-A₁-F/R. The products were digested with Kpn I and EcoR I, and subsequently inserted into the Kpn I and EcoR I digested pColdI to create plasmid pIM 3215. The intron-free *ftchyA-A*₂ was amplified by PCR using primer pairs pQ8ftchyA-A₂-F/R. The products were digested with EcoR I and Not I, and subsequently inserted into the EcoR I and Not I digested pQ8 (an expression vector with MBP tag on N-terminus) to yield plasmid pIM 3216. The intron-free $ftchyA-C_T$ was amplified by PCR using primer pairs pColdI-*ftchyA*- C_T -F/R. The products were digested with *BamH* I and EcoR I, and then cloned into pColdI to yield plasmid pIM 3217. The PCR products were amplified by PCR using primer pairs pColdI-*ftchyA* ΔC_T -F/R and digested with Spe I and EcoR I. The products were cloned into the Spe I and EcoR I digested pIM 3208 to generate plasmid pIM 3218. The plasmid pIM 3218 was used to purify the C_T domain-truncated ftChyA Δ C_T (A₁-T₁-C₁-A₂-T₂) from *E. coli* BL21. To express the ftChyA-A₁T₁ in E. coli BL21, the intron-free ftchyA-A₁T₁ was amplified by PCR using primer pairs pColdI-ftchyA-A₁-F/pColdI-ftchyA-A₁T₁-R. The products were digested with Kpn I and EcoR I, and then cloned into pColdI to yield plasmid pIM 3219.

To express *NpgA* without N-His tag in *E. coli* BL21, intron-free *NpgA* was cloned from the cDNA of *A. nidulans* by using the primer pairs pET28a-*NpgA*-F/R. The PCR products were digested with *Hind* III and *Nco* I and subsequently inserted into the *Hind*

III and Nco I digested pET28a to yield plasmid pIM 3220. To express NpgA with N-His tag, intron-free NpgA was cloned using the primer pairs pColdI-NpgA-F/R. The PCR products were digested with Sac I and Hind III and subsequently inserted into pColdI to yield plasmid pIM 3221. The plasmid pIM 3222 used for protein expression ftchyC in E. coli BL21 was constructed by amplifying open reading frames from cDNA reverse transcribed from A. nidulans containing ftchy cluster using primer pairs pColdIftchyC-F/R. The PCR products were digested with Kpn I and EcoR I and subsequently inserted into the Kpn I and EcoR I digested pColdI to yield plasmid pIM 3222. Intronfree ftchyD was cloned from the cDNA of A. nidulans containing ftchy cluster using the primer pairs pET-Duet-ftchyD-F/R. The PCR products were digested with BamH I and Not I, and subsequently inserted into the BamH I and Not I digested pET-Duet (an expression vector with His tag on N-terminus) to yield plasmid pIM 3223. Intron-free ftchyE was amplified by PCR using primer pairs pQ8-ftchyE-F/R. The PCR products were digested with EcoR I and Not I and subsequently inserted into the EcoR I and Not I digested pQ8 to yield plasmid pIM 3224. To express ftchyM in E. coli BL21, intronfree *ftchyM* was cloned using the primer pairs pColdI-*ftchyM*-F/R. The PCR products were digested with Kpn I and BamH I and subsequently inserted into the Kpn I and BamH I digested pColdI to yield plasmid pIM 3225. The intron-free ftchyH was amplified by PCR using primer pairs pColdI-ftchyH-F/R. The fragments were digested with Kpn I and BamH I and subsequently inserted into the Kpn I and BamH I digested pColdI to yield plasmid pIM 3226. The *ftchyH* was also cloned into the *EcoR* I and *Not* I digested pQ8 and pGEX-4t-1 (an expression vector with Glutathione S-transferase tag on N-terminus) to create plasmids pIM 3227 and pIM 3228, respectively.

The plasmid used for protein expression of *ftchyH* in *S. cerevisiae* BJ5464-NpgA was constructed by amplifying open reading frames from *A. nidulans* containing *ftchy* cluster using primer pairs pYEU-*ftchyH*-F/pYEU-*ftchyH*-R. The fragments were ligated to vector pYEU (an expression vector with Flag tag on N-terminus and His tag on C-terminus) with the promoter ADH₂P and the terminator ADH₂T by yeast homologous recombination to obtain plasmid pIM 3229.

All the plasmids were confirmed by DNA sequencing by Sangon Biotech (Shanghai) Co., Ltd.

1.4 The protein expression and purification in E. coli

Recombinant plasmids pIM 3208, pIM 3212-3218 and pIM 3221-3228 were transformed into *E. coli* BL21 (DE3) strain by heat shock transformation, respectively. The mono colony was cultivated in liquid LB medium (25 g/L LB broth) with 100 μ g/mL ampicillin (for ftChyA and its mutants, NpgA, ftChyC, ftChyH-N-His, ftChyH-N-GST, ftChyA-A₁, ftChyA-C_T, ftChyM, ftChyD) or 50 μ g/mL of kanamycin (for ftChyH-N-MBP, ftChyE, ftChyA-A₂) at 37 °C overnight. The bacterial solution was then transferred in liquid LB medium (1 L) containing 100 μ g/mL ampicillin or 50 μ g/mL of kanamycin at 37 °C and 220 rpm to OD₆₀₀ of 0.4-0.6. And then, the cultures were maintained at 16 °C for 30 min. Protein expression was induced at 16 °C and 220

rpm for 20 h after adding 0.2 mM isopropylthio- β -D-galactoside (IPTG). Cells were collected by centrifugation at 4 °C, 3000 g for 8 min.

The cells of ftChyA were resuspended in 20 mL buffer A (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 7.5) and were lysed by sonication on ice. Cellular debris were removed by centrifugation at 4 °C, 23000 g for 40 min. The protein was purified by nickel nitrilotriacetic acid (Ni-NTA) agarose resin. And then, the protein was eluted by buffer A containing 250 mM imidazole. The purified protein was passed through a PD-10 desalting column (GE Healthcare) and eluted with buffer C (50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 7.5). The protein was concentrated using ultrafiltration centrifugal tube (Millipore Amicon ® Ultra-15 mL). Finally, the protein was flash frozen in liquid nitrogen and saved at -80 °C. ftChyA mutants, NpgA, ftChyC, ftChyA-A₁, ftChyA-A₁T₁, ftChyA-C_T, ftChyA/C_T and ftChyD were treated in the same way. ftChyM was also treated in the same way, but it was resuspended in buffer A containing 1 mM DTT (DL-Dithiothreitol) and eluted buffer containing 250 mM imidazole and 1mM DTT.

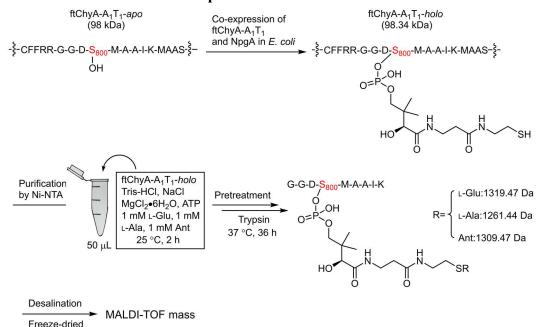
ftChyA-A₂ and ftChyE were expressed with N-MBP tag, respectively. The cells of ftChyA-A₂ or ftChyE were resuspended in 20 mL binding buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) and were lysed by sonication on ice. Cellular debris were removed by centrifugation at 4 °C, 23000 g for 40 min. The protein was purified using dextrin sepharose resin. The protein-bound resin was washed with elution buffer (10 mM maltose in binding buffer). The pooled fraction was concentrated and was exchanged into buffer C.

The purified enzyme was analyzed by SDS-PAGE, and the concentration was measured by BCA protein quantification kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd).

1.5 The preparation and transformation of A. nidulans protoplasts

A. nidulans was cultured in solid CD medium containing 10 mM uridine, 5 mM uracil, 1 µg/mL pyridoxine HCl and 0.25 µg/mL riboflavin at 37 °C for 4 days, and then spores were collected in 20% glycerol. The spores were inoculated in 40 mL liquid CD medium and cultured at 37 °C and 220 rpm for 9 h. After the germination of spores, cultures were centrifuged at 4 °C, 2500 g for 8 min to harvest the mycelia. The precipitation was washed two times with 15 mL osmotic buffer (1.2 M MgSO₄·7H₂O, 10 mM sodium phosphate, pH 5.8) at 4 °C, 2500 g for 8 min. Then the precipitation was resuspended with 10 mL osmotic buffer containing 30 mg Lysing Enzymes (Sigma) and 20 mg Yatalase (Takara). The suspension was transferred into 50 mL Erlenmeyer flask, and cultured at 28 °C, 80 rpm for 14 h. The culture fluid was poured directly in a sterile 50 mL centrifugal tube and overlaid gently with 10 mL of trapping buffer (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7.0), then centrifuged at 4 °C, 3000 g for 8 min. The protoplasm layer was transferred and fully scattered into 2 × STC buffer (1.2 M sorbitol, 10 mM CaCl₂, 10 mM Tris-HCl, pH 7.5), and centrifuged at 4 °C, 3000 g for 8 min. The supernatant was removed and STC buffer was added to resuspend the protoplasts

for transformation.



1.6 MALDI-TOF mass data acquisition

To gain *holo* form of ftChyA-A₁T₁, the recombinant plasmids pIM 3219 and pIM 3220 were co-transformed into *E. coli* BL21 (DE3) strain. The mono colony with *ftchyA-A₁T₁* and *NpgA* was selected in solid LB medium with 100 µg/mL ampicillin and 50 µg/mL of kanamycin at 37 °C overnight. Protein induction and co-expression of ftChyA-A₁T₁ and NpgA as described above. The protein of ftChyA-A₁T₁-*holo* was purified by Ni-NTA agarose resin. The 50 µL reaction mixture containing 40 µM ftChyA-A₁T₁-*holo*, 20 mM Tris-HCl (pH 8.5), 100 mM NaCl, 10 mM MgCl₂·6H₂O, 4 mM ATP, 1 mM L-Glu, 1 mM L-Ala and 1 mM Ant for 2 hours at 25 °C.

To analysis of ftChyA-A1 recognized and loaded amino acid to the Ser binding site (S_{800}) of ftChyA-T₁ by MALDI-TOF mass, the following steps will be performed. The 50 µL reaction mixture and 150 µL urea solution (8 M) were added to a 10 kDa ultrafiltration centrifugal tube, and then centrifuged at room temperature (RT), 12,000 g for 20 min. 200 µL urea solution (8 M) was added and centrifuged, which process will be repeated twice. 4 µL DTT (1 M) and 150 µL urea solution (8 M) were added and the nozzle of tube was sealed with parafilm, which incubated at 37 °C for 2 h. Next, 15 µL iodoacetamide solution (1 M) was added and incubated at RT for 1 h in the dark, subsequently centrifuged at RT, 12,000 g for 20 min. 200 µL urea solution (8 M) was added and centrifuged, which process will be repeated twice. 200 µL NH4HCO3 solution (50 mM) was added and centrifuged, which process will be repeated three times. The outer tube of the ultrafiltration centrifugal tube was replaced with a new outer tube. 200 µL trypsin solution (10 µg/mL) was added and the nozzle of tube was sealed with parafilm, which incubated at 37 °C for 36 h to decompose the protease into peptide fragments. The parafilm was removed and centrifuged. 40 µL NH4HCO3 solution (50 mM) was added and centrifuged. The lyophilized peptide sample was freeze-dried at 4°C. The sample was reconstituted with 200 μ L 0.1% TFA (trifluoroacetic acid) and centrifuged with a transient. A Zip Tip C18 micro desalting column was used for desalting peptide fragments. Elution buffer (90% acetonitrile (ACN) with 0.1% TFA) was used to elute the peptide, which was freeze-dried at 4 °C. MALDI-TOF mass data was recorded by a Bruker MALDITOF mass spectrometer Autoflex Speed and α -cyano-4-hydroxycinnamic acid (CHCA) (Sigma-Aldrich) was used as the matrix for intact precursor peptide. In general, the sample dissolved in 50 μ L 0.1% MeOH, and then 2 μ L sample was spotted and dried on the target. Subsequently, 2 μ L matrix solution (5 mg/mL CHCA dissolved in 50% ACN and 0.1% TFA) was spotted on top of the sample. MALDI-TOF mass data was acquired using the following parameters. Tuning: reflectron; Mass range: 800-2000; Laser diameter: 100 μ m; Laser power: 80-100 mV. Cytochrome C and TOFMixTM (SHIMADZU) was used for calibration.

1.7 Bioinformatic analysis

To obtain *ftchy* gene cluster from *F. tricinctum* CGMCC 3.4731, we used *chyA* protein sequence from *P. chrysogenum* to retrieve *chyA* orthologues by using localblast in public fungal genome from NCBI (National Center for Biotechnology Information) database and the private database of our lab. For gene cluster annotation of *ftchy*, 2ndFind program was used to predict the open reading frame and intron. The gene function was assigned based on NCBI Blast search. The domains of ftChyA were analyzed by *interpro* website. Active sites analyses of the ftChyA-C₁, ftChyA-C_T, ftChyE, ftChyD and ftChyM were performed using DNAMAN8.0 software, respectively. The conserved domain analysis of ftChyM and ftChyH were performed using NCBI Blast.

To analyze the phylogenetic relationships of ftChyA-C₁ and ftChyA-C_T domain, multiple sequence alignment of the ftChyA-C₁ and ftChyA-C_T domain sequences and other C domain sequences (TqaA, AmpA, AmpB, BenZ, PsyA, RoqA, IvoA, SimA, Aba1, EasA, FGSG_08209, FPSE_09183, Glip, Sirp, HasD, NFIA_064400, PENFLA_c013G03821) obtained from NCBI database were performed using ClustalW. Evolutionary analyses were conducted in MEGA7 software, and the phylogenetic tree was inferred by using neighbor-joining method.

TqaA: ADY16697.1, https://www.ncbi.nlm.nih.gov/protein/ADY16697.1.

AmpA: A0A1W6BT53.1, https://www.ncbi.nlm.nih.gov/protein/A0A1W6BT53.1.

AmpB: A0A1W6BT46.1, https://www.ncbi.nlm.nih.gov/protein/A0A1W6BT46.1.

BenZ: P9WEU9.1, https://www.ncbi.nlm.nih.gov/protein/P9WEU9.1.

PsyA: AMQ36132.1, https://www.ncbi.nlm.nih.gov/protein/AMQ36132.1.

RoqA: B6HJU6.1, https://www.ncbi.nlm.nih.gov/protein/B6HJU6.1.

IvoA: C8V7P4.1, https://www.ncbi.nlm.nih.gov/protein/C8V7P4.1.

SimA: CAA82227.1, https://www.ncbi.nlm.nih.gov/protein/CAA82227.1.

Aba1: ACJ04424.1, https://www.ncbi.nlm.nih.gov/protein/ACJ04424.1.

EasA: C8VPS9.1, https://www.ncbi.nlm.nih.gov/protein/C8VPS9.1.

FGSG_08209: I1RVD9.1, https://www.ncbi.nlm.nih.gov/protein/I1RVD9.1.

FPSE_09183: K3VDP2.1, https://www.ncbi.nlm.nih.gov/protein/K3VDP2.1.

Glip: Q4WMJ7.1, https://www.ncbi.nlm.nih.gov/protein/Q4WMJ7.1.

Sirp: Q6Q883.1, <u>https://www.ncbi.nlm.nih.gov/protein/Q6Q883.1</u>.

HasD: XP_754329.2, https://www.ncbi.nlm.nih.gov/protein/XP_754329.2.

NFIA_064400: XP_001263173.1, <u>https://www.ncbi.nlm.nih.gov/protein/XP_0012631</u>73.1.

PENFLA_c013G03821: OQE22222.1, <u>https://www.ncbi.nlm.nih.gov/protein/OQE22</u>222.1.

The enzyme function initiative-enzyme similarity tool (EFI-EST) was used to generate protein sequence similarity networks (SSNs) of ftChyM with fungal α -ketoglutarate dependent dioxygenases or the ftChyA-C₁ and ftChyA-C_T with other fungal C domains. Cytoscape (v3.8.2) software was used for visualizing complex networks of ftChyM or ftChyA-C₁ and ftChyA-C_T.

2nd Find: http://biosyn.nih.go.jp/2ndFind/.

NCBI BLAST: <u>https://blast.ncbi.nlm.nih.gov/Blast.cgi.</u>

interpro website: <u>http://www.ebi.ac.uk/interpro/search/sequence/.</u>

initiative-enzyme similarity tool (EFI-EST): https://efi.igb.illinois.edu/efi-est/.

1.8 Purification and structural characterization of compounds

To isolate 1, 4, 5, 6, 7, 8 and 9, the AN-ftchyACDEHM transformants were cultured in 16 L solid CD-ST medium at 25 °C for 4 days. And then, the culture was extracted with ethyl acetate/acetone (v/v, 3/1) for three times. The organic extracts were evaporated to dryness. The extracts were separated by Medium pressure liquid chromatography (MPLC) Reveleris® X2 (BUCHI, Switzerland) with a linear gradient of 10% MeOH-H₂O for 10 min, 10%-50% MeOH-H₂O in 40 min, 50%-100% MeOH-H₂O in 15 min, followed by 100% MeOH-H₂O for 15 min with flow rate of 30 mL/min on C18 column. The fractions containing 1 further separated by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; solvent: MeOH-H₂O, 35:65; flow: 2.5 mL/min; detector: 210 nm; $t_{\rm R}$ = 21.0 min) to yield 1 (10 mg). The compound 4 was purified by HPLC (column: YMC-Pack ODS-A, 5 µm, 10 × 250 mm; solvent: MeCN-H₂O (water with 0.1% v/v formic acid), 26:74; flow: 2.5 mL/min; detector: 210 nm; $t_{\rm R}$ =18.0 min) to give 4 (2 mg). The compound 5 was purified by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; solvent: MeCN-H₂O, 10:90; flow: 2.5 mL/min; detector: 210 nm) to give 5 (4 mg, $t_{\rm R}$ = 35.0 min). The compound 6 was further purified by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 \times 250 mm; solvent: MeOH-H₂O (water with 0.1% v/v formic acid), 42:58; flow: 2.5 mL/min; detector: 210 nm; $t_{\rm R}$ = 30.0 min) to yield 6 (3.7 mg). The compound 7 further purified by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 µm, 10 × 250 mm; solvent: MeOH-H₂O, 18:82; detector: 265 nm) to give 7 (12 mg, $t_R = 33.0$ min). The compound 8 (8.8 mg) was isolated using 11 % (v/v) MeCN-H₂O as the mobile phase by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; flow: 2.5 mL/min; detector: 210 nm; $t_{\rm R}$ =45.0 min). The compound 9 (10 mg) was separated by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; MeCN-H₂O, 10:90; flow: 2.5 mL/min; detector: 210 nm; t_R =24.0 min).

To isolate 10, the AN-ftchyA transformants were cultured in 4.8 L solid CD-ST medium containing 10 mM uridine, 5 mM uracil and 0.25 µg/mL riboflavin at 25 °C for 4 days. And then, the culture was extracted with MeOH for three times. The extracts were evaporated to dryness. The extracts were separated by Medium pressure liquid chromatography (MPLC) Reveleris® X2 (BUCHI, Switzerland) with a linear gradient of 10% MeOH-H2O for 10 min, 10%-50% MeOH-H2O in 30 min, 50%-100% MeOH-H₂O in 20 min, followed by 100% MeOH-H₂O for 20 min with flow rate of 25 mL/min on C18 column. The fractions containing 10 further separated by semi-preparative HPLC (column: YMC-Pack ODS-A, 5 µm, 10 × 250 mm; solvent: MeOH-H₂O (water with 0.1% v/v formic acid), 40:60; flow: 2.5 mL/min; detector: 210 nm; $t_{\rm R}$ = 18.0 min) to yield **10** (12 mg). ¹H NMR (400 MHz, DMSO- d_6): δ 12.25 (s, 1H), 8.64 (d, J = 6.5Hz, 1H), 8.53 (dd, *J* = 8.6, 1.0 Hz, 1H), 7.94 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.42 (td, *J* = 7.9, 1.7 Hz, 1H), 7.06 (td, J = 7.6, 1.2 Hz, 1H), 4.20 (m, 1H), 3.64 (dd, J = 9.4, 4.7 Hz, 1H), 2.54 (m, 1H), 2.45 (m, 1H), 2.18 (m, 1H), 1.82 (m, 1H), 1.35 (d, J = 7.4 Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 171.7, 171.5, 171.4, 169.4, 139.3, 131.7, 131.3, 122.3, 122.0, 118.9, 52.0, 50.4, 29.9, 26.0, 17.3; UV/Vis: 221, 252, 302 nm; HRMS (m/z): $[M]^+$ calcd. for C₁₅H₂₀N₃O₆, 338.1347; found, 338.1346.

To isolate **3**, the large-scale *in vitro* biochemical assays of ftChyA with L-Ala and Ant were performed. The purified 10 μ M ftChyA was converted to its *holo* form by incubation in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 μ M NpgA, 0.1 mM CoA and 10 mM MgCl₂·6H₂O in buffer C (pH 7.5) (total volume 52 mL) at 25 °C for 1 h. Reactions were initiated by the addition of 5 mM ATP, 1 mM Ant and 1 mM L-Ala. The reaction mixture was incubated at 25 °C overnight and then freeze-dried. The products were dissolved in MeOH and purified by semipreparative HPLC with a C18 column (YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; solvent: MeOH-H₂O (water with 0.02% v/v formic acid), 25:75; flow: 2.5 mL/min; detector: 210 nm; *t*_R = 12.5 min; 2 mg).

To isolate **11** and **2**, the large-scale *in vitro* biochemical assays of ftChyD with **10** and **3** were performed, respectively. The reaction mixture containing 20 μ M ftChyD, 200 μ M **10**, 10 mM MgCl₆·H₂O, 4 mM ATP, 2 mM L-Gln and 20 mM Tris-HCl (pH 7.5) in buffer C (pH 7.5) (total volume 75 mL) at 25 °C overnight. The reaction mixture containing 20 μ M ftChyD and 200 μ M **3** in buffer C (pH 7.5) (total volume 120 mL) under the same conditions. The reaction mixture was freeze-dried, and the products were dissolved in MeOH. Compound **11** was purified by semipreparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; solvent: MeOH-H₂O (water with 0.1% v/v formic acid), 20:80; flow: 2.5 mL/min; detector: 210 nm; t_R = 22.5 min) to yield **11** (4.1 mg). ¹H NMR (400 MHz, DMSO- d_6): δ 11.94 (s, 1H), 8.71 (d, *J* = 6.6 Hz, 1H), 8.48 (dd, *J* = 8.4, 1.2 Hz, 1H), 8.27 (s, 1H), 7.91 (s, 1H), 7.77 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.48 (td, *J* = 8.0, 1.5 Hz, 1H), 7.11 (td, *J* = 7.6, 1.2 Hz, 1H), 4.20 (m, 1H), 3.30 (t, *J* = 6.2 Hz, 1H), 2.44 (t, *J* = 7.7 Hz, 2H), 1.98 (m, 1H), 1.90 (m, 1H), 1.32 (d, *J* = 7.3

Hz, 3H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 172.6, 171.6, 170.7, 170.0, 139.1, 132.0, 128.5, 122.4, 120.2, 119.9, 53.8, 50.2, 31.7, 26.6, 17.2; UV/Vis: 212, 250, 293 nm; HRMS (m/z): [M]⁺ calcd. for C₁₅H₂₁N₄O₅, 337.1506; found, 337.1506.

Compound **2** was separated by semipreparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; MeOH-H₂O (water with 0.02% v/v formic acid), 5:95; flow: 2.5 mL/min; detector: 210 nm) to yield **2** (3.5 mg, *t*_R=12.5 min).

To isolate 14 from large-scale spontaneous conversion of 2 in Tris-HCl buffer (pH 7.5). Compound 2 (3 mg) was added to 500 mL buffer C (pH 7.5). The reaction mixture was kept at 25 °C for 36 h and extracted with ethyl acetate 4 times. The ethyl acetate layer was evaporated until dry and separated by semipreparative HPLC (column: YMC-Pack ODS-A, 5 μ m, 10 × 250 mm; solvent: MeCN-H₂O (water with 0.1% v/v formic acid), 12:88; flow: 2.5 mL/min; detector: 210 nm; $t_R = 10$ min) to yield 14 (1.6 mg).

2. Supplementary Tables

Supplementary Table 1. Plasmids used in this study.

Name	Description	Enzyme site	Aim
pIM 3201	ftchyA gDNA with downstream 387 bp, in	BamH I	A. nidulans
	pANR		overexpression
pIM 3202	ftchyC gDNA with downstream 425 bp,	Not I	A. nidulans
	ftchyD gDNA with downstream 264 bp,		overexpression
	ftchyE gDNA with downstream 395 bp, in		
	pANU		
pIM 3203	ftchyM gDNA with downstream 267 bp,	BamH I	A. nidulans
	ftchyH gDNA with downstream 426 bp,		overexpression
	in pANP		
pIM 3204	ftchyC gDNA with downstream 425 bp,	Not I	A. nidulans
•	ftchyD gDNA with downstream 264 bp, in		overexpression
	pANU		•
pIM 3205	<i>ftchyH</i> gDNA with downstream 426 bp,	BamH I	A. nidulans
1	in pANP		overexpression
pIM 3206	<i>ftchyM</i> gDNA with downstream 267 bp,	BamH I	A. nidulans
p101 5 200	in pANP	Dumii i	overexpression
pIM 3207	<i>ftchyA</i> cDNA in pYEU with Flag and His tag	Spe I-Pml I	To provide <i>ftchyA</i> gene
pIM 3207	<i>ftchyA</i> cDNA in pColdI with His tag	BamH I-EcoR I	<i>E. coli</i> overexpression
-			
pIM 3209	<i>ftchyA-C1-H987A</i> cDNA in pYEU with Flag	Spe I-Pml I	To provide <i>ftchyA</i> - C_{l} -
71.0010	and His tag	<i>a</i>	H ₉₈₇ A gene
pIM 3210	<i>ftchyA-CT-H2074A</i> cDNA in pYEU with Flag	Spe I-Pml I	To provide <i>ftchyA-C</i> _T -
	and His tag		H2075A gene
pIM 3211	$ftchyA-C_1^*$ (A ₉₈₆ A ₉₈₇ xxxA ₉₉₁) cDNA in	Spe I-Pml I	To provide <i>ftchyA</i> - C_1^*
	pYEU with Flag and His tag		gene
pIM 3212	ftchyA-C1-H987A cDNA in pColdI with His	BamH I-EcoR I	To prove the active
	tag		residues of ftChyA-C1
			domain
pIM 3213	ftchyA-CT-H2074A cDNA in pColdI with His	BamH I-EcoR I	To prove the active
	tag		residues of ftChyA-CT
			domain
pIM 3214	$ftchyA-C_1^*$ (A986A987xxxA991) cDNA in	BamH I-EcoR I	To prove the active
	pColdI with His tag		residues of ftChyA-C1
			domain
pIM 3215	<i>ftchyA-A</i> ¹ cDNA in pColdI with His tag	Kpn I-EcoR I	E. coli overexpression
•		*	ftChyA-A1 domain
			alone
pIM 3216	<i>ftchyA-A</i> ² cDNA in pQ8 with MBP tag	EcoR I-Not I	E. coli overexpression
r	,,		ftChyA-A ₂ domain
			alone
pIM 3217	<i>ftchyA-C</i> ^T cDNA in pColdI with His tag	BamH I-EcoR I	<i>E. coli</i> overexpression
pini 5217	Jenyn er ebint in peolar with this ag	Bumii i Ecolt i	ftChyA-C _T domain
			alone
pIM 3218	<i>ftchyA</i> ΔC_T cDNA in pColdI with His tag	BamH I-EcoR I	To prove the ftChyA-C _T
PIN 3210	JUNYALICT CONA III POOLAI WILLI HIS LAG	Dumii I-ECON I	
IM 2210	Ashid A.T. (DNA in C.111 id 11')		domain
pIM 3219	$ftchyA-A_1T_1$ cDNA in pColdI with His tag	Kpn I-EcoR I	<i>E. coli</i> overexpression
D (2222			ftChyA-A ₁ T ₁ domain
pIM 3220	NpgA cDNA in pET28a without His tag	Hind III-Nco I	E. coli overexpression

pIM 3221	NpgA cDNA in pColdI with His tag	Sac I-Hind III	E. coli overexpression
pIM 3222	ftchyC cDNA in pColdI with His tag	Kpn I-EcoR I	E. coli overexpression
pIM 3223	ftchyD cDNA in pET-Duet with His tag	BamH I-Not I	E. coli overexpression
pIM 3224	ftchyE cDNA in pQ8 with MBP tag	EcoR I-Not I	E. coli overexpression
pIM 3225	ftchyM cDNA in pColdI with His tag	Kpn I-BamH I	E. coli overexpression
pIM 3226	ftchyH cDNA in pColdI with His tag	Kpn I-Hind III	E. coli overexpression
pIM 3227	ftchyH cDNA in pQ8 with MBP tag	EcoR I-Not I	E. coli overexpression
pIM 3228	ftchyHcDNA in pGEX-4t-1 with GST tag	EcoR I-Not I	E. coli overexpression
pIM 3229	ftchyH cDNA in pYEU with Flag and His	Spe I-Pml I	S. cerevisiae
	tag		overexpression

Products	Chemical Formula	Molecular Weight	KO chyC	KO chyE	KO chyH
	C13H13N3O4	275	V		V
	C12H15N3O3	249	\checkmark	\checkmark	\checkmark
	C13H15N3O5	293	\checkmark	\checkmark	\checkmark
	C13H14N2O6	294	\checkmark	\checkmark	7
	C15H20N4O5	336		V	V
	C15H19N3O6	337	V	V	V
NH NH ÖH	$C_{10}H_{10}N_2O_2$	190	\checkmark	V	V

Supplementary Table 2. Products from gene (*chyC*, *chyE* and *chyH*) individual KO mutant of *P*. *chrysogenum*¹.

Gene	Size(bp)	Gene function	P. chrysogenum (identity/similarity)	<i>F. graminearum</i> (identity/similarity)
ftchyA	7274	NRPS (A ₁ -T ₁ -C ₁ -A ₂ -T ₂ -C _T)	64/76	64/78
ftchyC	786	short-chain dehydrogenase (SDR)	79/87	82/91
ftchyD	2287	class II amidotransferase	80/89	87/95
ftchyE	824	glutaminase	31/47	56/74
ftchyH	1743	flavin-dependent oxidase	69/82	74/84
ftchyM	1179	α-KG dioxygenase	71/82	73/83

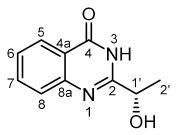
Supplementary Table 3. Analysis of *ftchy* gene cluster from *F. tricinctum*.

	2	Ion	Meas. m/z	Meas. m/z	Calc. m/z	Calc. m/z	Err
Com.	Structure	Formula	$[M+H]^+$	[M+Na] ⁺	$[M+H]^+$	[M+Na] ⁺	(ppm)
1		C10H11N2O2	191.0815		191.0815		0.1
2	NH ₂ NH	C ₁₀ H ₁₄ N ₃ O ₂	208.1081		208.1081		-0.3
3	O NH O NH ₂	C10H13N2O3	209.0921		209.0921		-0.4
4	NH ₂ NH O	C10H10N2NaO3		229.0584		229.0584	0.0
5	NH ₂ NH	$C_{10}H_{12}N_2NaO_3$		231.0741		231.0740	-0.3
6	NH NH O	C10H9N2O2	189.0655		189.0659		2.0
7		C12H15N3NaO3		272.1007		272.1006	-0.3
8		$C_{12}H_{14}N_{3}O_{2}$	232.1082		232.1081		-0.8
9	NH ₂	C7H9N2O	137.0706		137.0709		2.8

Supplementary Table 4. HRMS data of compounds in this study.

10	H_2N	C ₁₅ H ₂₀ N ₃ O ₆	338.1346		338.1347		0.2
11		C15H21N4O5	337.1506		337.1506		0.2
14	NH N N N NH	C10H12N3O	190.0977		190.0975		-0.9
Ant-Me-3		C11H15N2O3	223.1077		223.1077		0.1
Ant-Me- 10		C16H21N3NaO6		374.1323		374.1323	-0.1

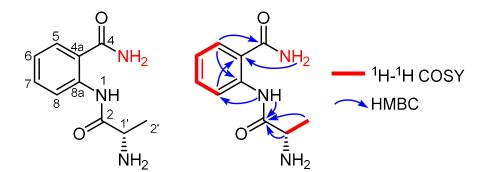
Supplementary Table 5. NMR data of compound 1 in DMSO-d₆.



(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

NO.	δc, type ²	δc , type	$\delta_{\rm H}$ (mult, <i>J</i> in Hz) ²	$\delta_{ m H}$ (mult, J in Hz)
1				
2	159.6, C	159.9, C		
3			11.79 (1H, br s, NH)	11.83 (1H, br s, NH)
4	161.4, C	161.7, C		
4a	121.2, C	121.2, C		
5	125.7, CH	125.8, CH	8.10 (1H, d, 7.6)	8.10 (1H, dd, 8.0,1.5)
6	126.2, CH	126.8, CH	7.48 (1H, t, 7.5)	7.48 (1H, t, 8.0)
7	134.3, CH	134.4, CH	7.79 (1H, t, 7.6)	7.79 (1H, td, 8.0,1.5)
8	126.9, CH	126.3, CH	7.63 (1H, d, 8.1)	7.64 (1H, d, 8.0)
8a	148.4, C	148.3, C		
1′	67.1, CH	67.2, CH	4.59 (1H, m)	4.59 (1H, q, 6.6)
2'	21.5, CH ₃	21.6, CH ₃	1.43 (3H, d, 6.6)	1.43 (3H, d, 6.6)
1'-OH			5.65 (1H, d, 7.6)	5.67 (1H, br s)

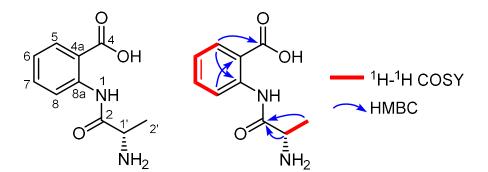
Supplementary Table 6. NMR data of compound 2 in DMSO-d₆.



	X .	,		,
NO.	$\delta_{ m H}$ (mult, J in Hz)	δc , type	COSY	НМВС
1	12.01 (1H, s)			C2, C8
2		167.9, C		
4		170.4, C		
4a		120.9, C		
5	7.84 (1H, dd, 7.9, 1.5)	128.7, CH	H6	C4, C7, C8a
6	7.20 (1H, td, 8.0, 1.2)	123.5, CH	H5, H7	C4a, C5, C8
7	7.55 (1H, td, 8.0, 1.5)	132.2, CH	H6, H8	C5, C8, C8a
8	8.32 (1H, dd, 8.3, 1.2)	120.8, CH	H7	C4a, C6
8a		138.3, C		
1′	4.18 (1H, q, 7.0)	49.3, CH	H2'	C2′, C2
2'	1.49 (3H, d, 7.0)	16.4, CH ₃	H1'	C1′, C2
1'-NH2	8.31 (2H, overlapped)			
4-NHa	7.79 (1H, s)			C4a
4-NH _b	8.31 (1H, overlapped)			

(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

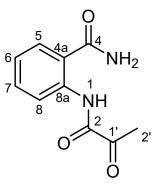
Supplementary Table 7. NMR data of compound **3** in DMSO-*d*₆.



NO.	$\delta_{ m H}$ (mult, J in Hz)	δc , type	COSY	HMBC
1	11.33 (1H, s)			
2		168.2, C		
4		169.0, C		
4a		118.9, C		
5	8.00 (1H, dd, 7.8, 1.2)	131.1, CH	H6	C4, C7, C8a
6	7.24 (1H, m)	123.8, CH	H5, H7	C4a
7	7.63 (1H, m)	133.7, CH	H6, H8	C5, C8a
8	8.26 (1H, dd, 8.4, 1.2)	121.1, CH	H7	C4a, C6
8a		139.0, C		
1′	4.22 (1H, q, 7.0)	49.3, CH	H2'	C2′, C2
2′	1.51 (3H, d, 6.8)	16.5, CH ₃	H1'	C1′, C2

(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

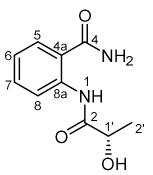
Supplementary Table 8. NMR data of compound 4 in DMSO-d₆.



(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

NO.	δc, type ³	δc , type	$\delta_{ m H}$ (mult, J in Hz) ³	$\delta_{ m H}$ (mult, J in Hz)
1			12.65 (1H, s)	12.66 (1H, s)
2	158.6, C	158.7, C		
4	170.2, C	170.3, C		
4a	120.6, C	120.7, C		
5	128.7, CH	128.8, CH	7.84 (1H, dd, 8.2, 1.2)	7.85 (1H, d, 8.0)
6	123.3, CH	123.4, CH	7.20 (1H, td, 8.2, 1.2)	7.21 (1H, t, 8.0)
7	132.2, CH	132.3, CH	7.56 (1H, td, 8.2, 1.2)	7.57 (1H, t, 8.0)
8	119.8, CH	119.8, CH	8.58 (1H, dd, 8.2, 1.2)	8.59 (1H, d, 7.8)
8a	138.0, C	138.1, C		
1′	196.3, C	196.4, C		
2'	24.1, CH ₃	24.1, CH ₃	2.43 (3H, s)	2.42 (3H, s)
4-NHa			8.28 (1H, br s)	8.31 (1H, br s)
4-NH _b			7.73 (1H, br s)	7.77 (1H, br s)

Supplementary Table 9. NMR data of compound 5 in DMSO-d₆.

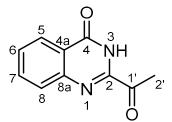


(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

NO.	δc, type ⁴	δc , type	$\delta_{ m H}$ (mult, J in Hz) ⁴	$\delta_{ m H}$ (mult, J in Hz)
1			11.88 (1H, s)	11.99 (1H, s)
2	174.7, C	174.1, C		
4	170.8, C	170.2, C		
4a	121.4, C	120.9, C		
5	128.8, CH	128.5, CH	7.70 (1H, dd, 7.8, 1.5)	7.74 (1H, dd, 7.8, 1.0)
6	123.2, CH	122.4, CH	7.11 (1H, t, 7.8)	7.11 (1H, td, 8.0, 1.0)
7	132.4, CH	131.8, CH	7.46 (1H, dt, 7.8, 1.5)	7.47 (1H, td, 8.0, 1.1)
8	120.4, CH	119.8, CH	8.45 (1H, d, 7.8)	8.57 (1H, d, 8.4)
8a	138.7, C	138.7, C		
1′	68.3, CH	67.9, CH	4.09 (1H, q, 7.0)	4.10 (1H, m)
2'	21.2, CH ₃	20.8, CH ₃	1.28 (3H, d, 7.0)	1.30 (3H, d, 6.8)
1'-OH			6.05 (1H, br s)	5.97 (1H, d, 5.0)
4-NHa			8.13 (1H, br s)	8.16 (1H, br s)
4-NH _b			7.05 (1H, br s)	7.60 (1H, br s)

.

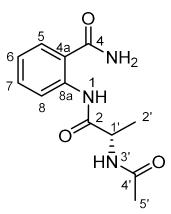
Supplementary Table 10. NMR data of compound 6 in DMSO-d₆.



(400 MHz for $^1\mathrm{H}$ NMR, 100 MHz for $^{13}\mathrm{C}$ NMR)

NO.	δc, type ⁵	δ c, type	$\delta_{\rm H}$ (mult, <i>J</i> in Hz) ⁵	$\delta_{ m H}$ (mult, J in Hz)
1				
2	147.1, C	147.2, C		
3			12.20 (1H, br s)	12.26 (1H, br s)
4	159.9, C	160.8, C		
4a	123.6, C	123.2, C		
5	126.1, CH	126.1, CH	8.20 (1H, m, H-Ar)	8.19 (1H, dd, 7.9, 1.5)
6	128.8, CH	128.9, CH		7.66 (1H, td, 8.2, 1.5)
7	134.7, CH	134.8, CH	7.40-7.90 (3H, m)	7.91 (1H, td, 8.2, 1.5)
8	128.5, CH	128.5, CH		7.85 (1H, dd, 7.9, 1.5)
8a	147.3, C	147.4, C		
1′	194.0, C	193.9, C		
2'	24.7, CH ₃	24.7, CH ₃	2.70 (3H, s)	2.64 (3H, s)

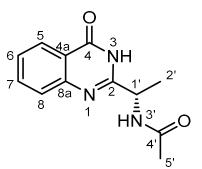
Supplementary Table 11. NMR data of compound 7 in DMSO-d₆.



(400 MHz for $^1\!\mathrm{H}$ NMR, 100 MHz for $^{13}\!\mathrm{C}$ NMR)

NO.	δc , type ⁶	δc , type	$oldsymbol{\delta}_{ m H}$ (mult, J in Hz) 6	$\delta_{ m H}$ (mult, J in Hz)
1			12.05 (1H, s)	12.09 (1H, s)
2	171.8, C	171.7, C		
4	170.7, C	170.6, C		
4a	119.7, C	119.6, C		
5	128.7, CH	128.6, CH	7.78 (1H, dd, 7.8, 1.3)	7.80 (1H, dd, 7.8, 0.8)
6	122.4, CH	122.4, CH	7.10 (1H, dd, 7.8, 0.8)	7.11 (1H, td, 7.8, 0.9)
7	132.3, CH	132.2, CH	7.48 (1H, dd, 7.9, 1.3)	7.49 (1H, td, 8.4, 1.2)
8	119.7, CH	119.7, CH	8.53 (1H, dd, 7.9, 0.8)	8.55 (1H, d, 8.4)
8a	139.5, C	139.5, C		
1′	50.1, CH	50.0, CH	4.20 (1H, dq, 7.3, 6.8)	4.21 (1H, m)
2'	17.3, CH ₃	17.3, CH ₃	1.31 (3H, d, 7.3)	1.31 (1H, d, 7.3)
3'			8.47 (1H, d, 6.8)	8.51 (1H, d, 6.8)
4′	169.8, C	169.7, C		
5'	22.7, CH ₃	22.6, CH ₃	1.94 (3H, s)	1.96 (3H, s)
4-NHa			8.23 (1H, br s)	8.26 (1H, br s)
4-NH _b			7.69 (1H, br s)	7.73 (1H, br s)

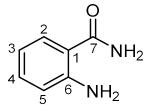
Supplementary Table 12. NMR data of compound 8 in DMSO-d₆.



(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

NO.	δc , type ⁷	δc , type	$\boldsymbol{\delta}_{\mathbf{H}}$ (mult, \boldsymbol{J} in Hz) ⁷	$\delta_{ m H}$ (mult, J in Hz)
1				
2	159.0, C	158.5, C		
3			12.16 (1H, s)	12.18 (1H, s)
4	162.1, C	161.6, C		
4a	121.6, C	121.1, C		
5	127.4, CH	126.9, CH	8.08 (1H, d, 8.0)	8.09 (1H, dd, 7.8, 1.0)
6	126.8, CH	126.3, CH	7.48 (1H, t, 7.5)	7.48 (1H, t, 7.3)
7	134.9, CH	134.4, CH	7.78 (1H, t, 7.5)	7.79 (1H, td, 7.8, 1.2)
8	126.3, CH	125.8, CH	7.61 (1H, d, 8.0)	7.62 (1H, d, 8.1)
8a	149.1, C	148.6, C		
1′	48.2, CH	47.7, CH	4.72 (1H, m)	4.72 (1H, m)
2'	19.7, CH ₃	19.2, CH ₃	1.38 (3H, d, 7.2)	1.39 (3H, d, 7.0)
3'			8.32 (1H, d, 7.05)	8.34 (1H, d, 7.0)
4′	169.8, C	169.3, C		
5'	23.0, CH ₃	22.5, CH ₃	1.87 (3H, s)	1.88 (3H, s)

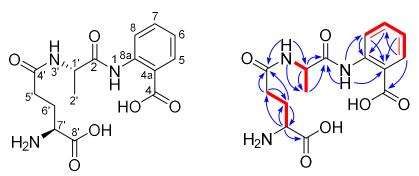
Supplementary Table 13. NMR data of compound 9 in DMSO-d₆.



(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

NO.	δc , type ⁸	δc , type	$\delta_{ m H}$ (mult, J in Hz) 8	$\delta_{ m H}$ (mult, J in Hz)
1	113.7, C	113.7, C		
2	150.1, CH	150.2, CH	6.67 (1H, dd, 7.8, 1.3)	6.67 (1H, dd, 8.2, 1.0)
3	114.3, CH	114.4, CH	7.12 (1H, td, 7.8, 1.3)	7.12 (1H, td, 8.0, 1.4)
4	131.8, CH	131.9, CH	6.47 (1H, td, 7.8, 1.3)	6.47 (1H, td, 8.2, 1.0)
5	116.3, CH	116.4, CH	7.52 (1H, dd, 7.8, 1.3)	7.52 (1H, dd, 8.0, 1.4)
6	128.7, C	128.7, C		
7	171.3, C	171.4, C		
-CONH _a			7.68 (1H, br s)	7.67 (1H, br s)
-CONH _b)		7.01 (1H, br s)	7.02 (1H, br s)
ArNH ₂			6.53 (2H, br s)	6.53 (2H, br s)

Supplementary Table 14. NMR data of compound 10 in DMSO-d₆.

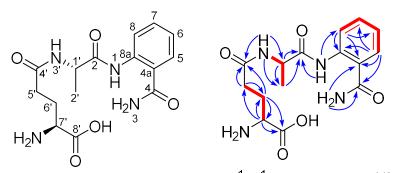


— ¹H-¹H COSY — HMBC

NO.	$\delta_{ m H}$ (mult, J in Hz)	δc , type	COSY	HMBC
1	12.25 (s)			C8, C4a, C2
2		171.5, C		
4		169.4, C		
4a		122.0, C		
5	7.94 (dd, 7.9, 1.7)	131.3, CH	H6	C7, C8, C4
6	7.06 (td, 7.6, 1.2)	122.3, CH	H7, H5	C8, C4a, C7, C8a
7	7.42 (td, 7.9, 1.7)	131.7, CH	H6, H8	C8, C6, C5, C8a
8	8.53 (dd, 8.6, 1.0)	118.9, CH	H7	C8a, C6, C5, C4a
8a		139.3, C		
1′	4.20 (m)	50.4, CH	H2′	C2′, C2
2'	1.35 (d, 7.4)	17.3, CH ₃		C1′, C2
3'	8.64 (d, 6.5)		H1′	C2', C1', C4'
4′		171.4, C		
5'a	2.54 (m)	29.9, CH ₂		C6', C7', C4'
5′b	2.45 (m)			C6', C7', C4'
6'a	2.18 (m)	$26.0,\mathrm{CH}_2$	H6′b, H5′a, H5′b	C7′, C4′, C5
6′b	1.82 (m)			C7′, C4′
7′	3.64 (dd, 9.4, 4.7)	52.0, CH	H6′a, H6′b	C6', C5', C8'
8'		171.7, C		

(400 MHz for 1 H NMR, 100 MHz for 13 C NMR)

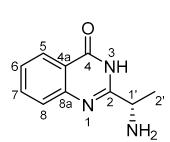
Supplementary Table 15. NMR data of compound 11 in DMSO-d₆



(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

NO.	$\delta_{ m H}$ (mult, J in Hz)	δc , type	COSY	НМВС
1	11.94 (s)			C8, C2
2		171.6, C		
3a	8.27 (s)			C4
3b	7.91 (s)			C4, C4a
4		170.7, C		
4a		120.2, C		
5	7.77 (dd, 8.0, 1.6)	128.5, CH	H6	C4a, C7, C8a, C4
6	7.11 (td, 7.6, 1.2)	122.4, CH	H7, H5	C4a, C5, C7
7	7.48 (td, 8.0, 1.5)	132.0, CH	H6, H8	C8, C5, C8a
8	8.48 (dd, 8.4, 1.2)	119.9, CH	H7	C4a, C6, C8a
8a		139.1, C		
1′	4.20 (m)	50.2, CH		C2′, C2
2'	1.32 (d, 7.3)	17.2, CH ₃	H1'	C2, C1′
3'	8.71 (d, 6.6)		H1'	C2', C1', C4'
4′		172.6, C		
5'	2.44 (t, 7.7)	31.7, CH ₂	H6′a, H6′b	C6', C7', C4'
6′a	1.98 (m)	$26.6,\mathrm{CH}_2$	H7′	C5', C7', C8', C4'
6'b	1.90 (m)		H7′	C5', C7', C8', C4'
7′	3.30 (t, 6.2)	53.8, CH		C6', C5', C8'
8'		170.0, C		

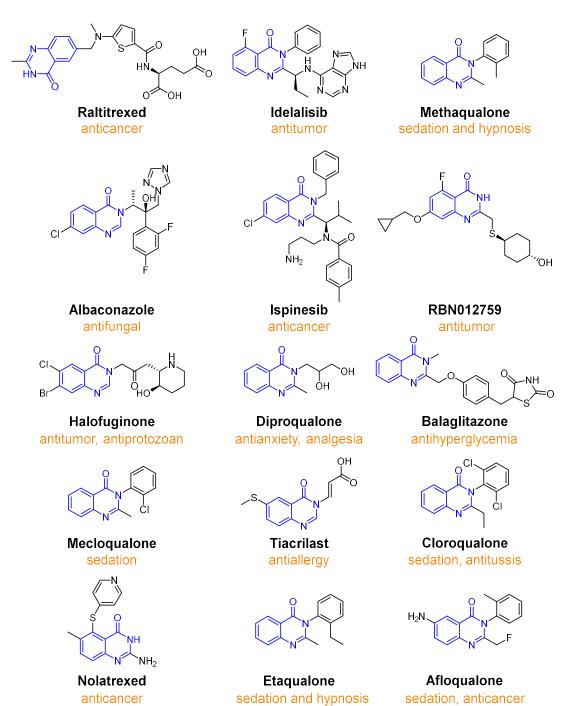
Supplementary Table 16. NMR data of compound 14 in DMSO-d₆.



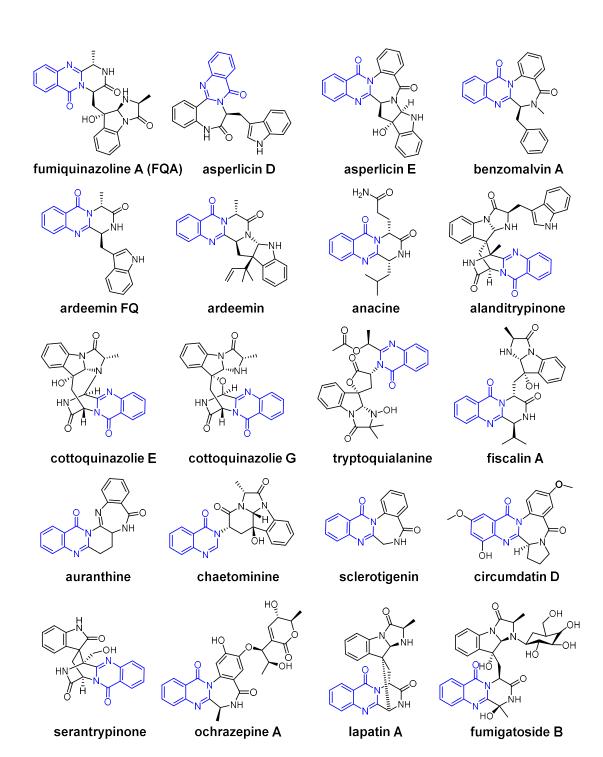
(400 MHz for ¹H NMR, 100 MHz for ¹³C NMR)

NO.	δc , type	$\delta_{ m H}$ (mult, J in Hz)
2	160.7, C	
4	161.8, C	
4a	121.1, C	
5	125.8, CH	8.10 (1H, m)
6	126.8, CH	7.50 (1H, m)
7	134.4, CH	7.80 (1H, m)
8	126.2, CH	7.63 (1H, m)
8a	148.5, C	
1′	49.8, C	3.90 (1H, m)
2'	21.5, CH ₃	1.36 (3H, s)
1'-NHa		8.26 (1H, br s)

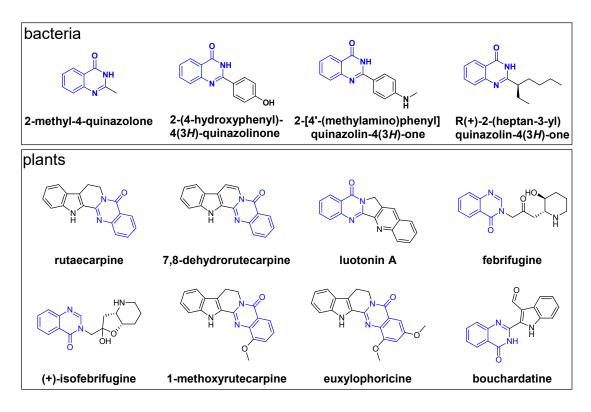
3. Supplementary Figures



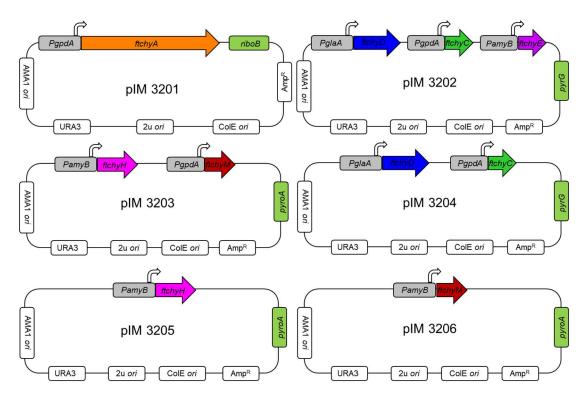
Supplementary Fig. 1 Marketed drugs with 4(3H)-quinazolinone scaffold.



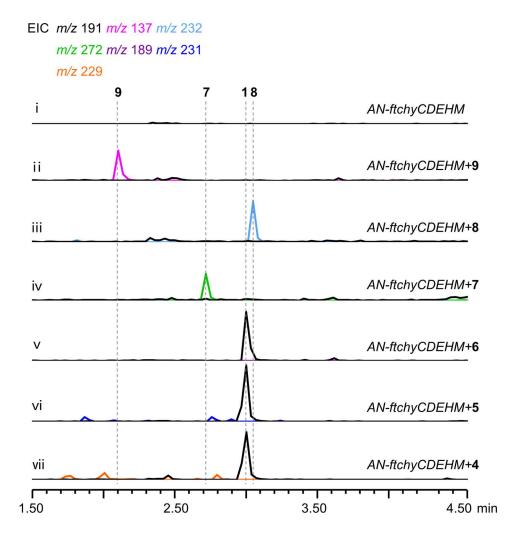
Supplementary Fig. 2 Representative natural 4(3*H*)-quinazolinone peptidyl alkaloids from fungi.



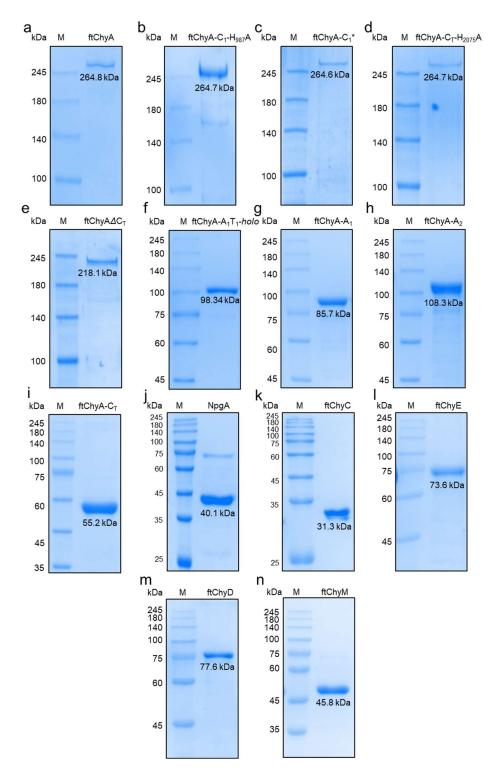
Supplementary Fig. 3 Representative natural 4(3H)-quinazolinone alkaloids from plants and bacteria.



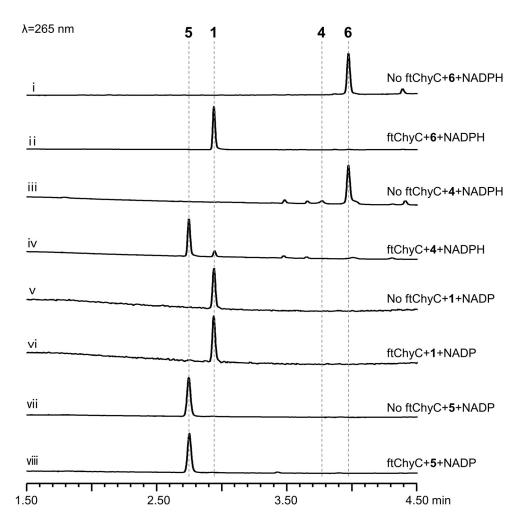
Supplementary Fig. 4 Construction of plasmids for overexpression of *ftchy* cluster in *A. nidulans*.



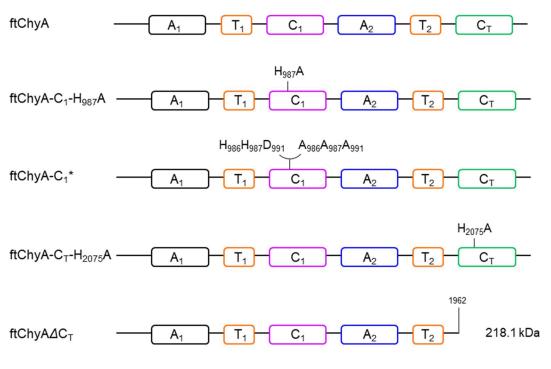
Supplementary Fig. 5 Confirmation of the on-pathway intermediates of 1 by chemical feeding experiments. The extracted ion chromatograms (EICs) were extracted at m/z 191 [M + H]⁺ for 1, m/z 229 [M + Na]⁺ for 4, m/z 231 [M + Na]⁺ for 5, m/z 189 [M + H]⁺ for 6, m/z 272 [M + Na]⁺ for 7, m/z 232 [M + H]⁺ for 8 and m/z 137 [M + H]⁺ for 9.



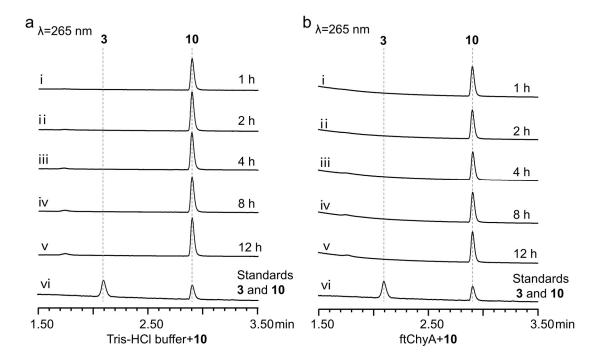
Supplementary Fig. 6 SDS-PAGE of proteins purified from *E. coli* BL21 in this study. (a) ftChyA (264.8 kDa). (b) ftChyA-C₁-H₉₈₇A (264.7 kDa). (c) ftChyA-C₁* (264.6 kDa). (d) ftChyA-C_T-H₂₀₇₅A (264.7 kDa). (e) ftChyA Δ C_T (218.1 kDa). (f) ftChyA-A₁T₁-*holo* (98.34 kDa). (g) ftChyA-A₁ (85.7 kDa). (h) ftChyA-A₂ (108.3 kDa) with N-MBP tag. (i) ftChyA-C_T (55.2 kDa). (j) NpgA (40.1 kDa). (k) ftChyC (31.3 kDa). (l) ftChyE (73.6 kDa) with N-MBP tag. (m) ftChyD (77.6 kDa). (n) ftChyM (45.8 kDa). M: marker. All experiments were repeated independently more than three times with similar results.



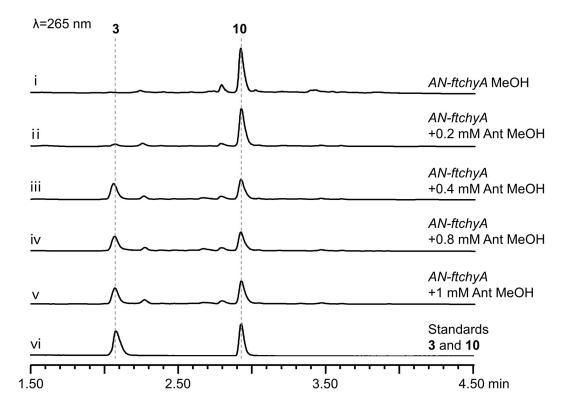
Supplementary Fig. 7 ftChyC-catalysed reduction or dehydrogenation reactions with different substrates. The spontaneous cyclization of **4** to form **6** is observed in control experiment.



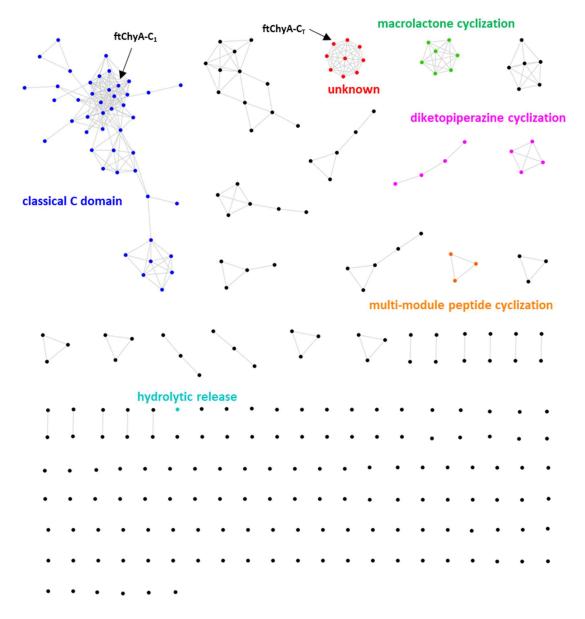
Supplementary Fig. 8 Schematic diagrams of ftChyA and its mutants.



Supplementary Fig. 9 Time-course assays of the stability of **10**. (**a**) in Tris-HCl buffer (pH 7.5), (**b**) with ftChyA addition.



Supplementary Fig.10 LC-MS analyses of the *AN-ftchyA* with feeding different concentrations of Ant.

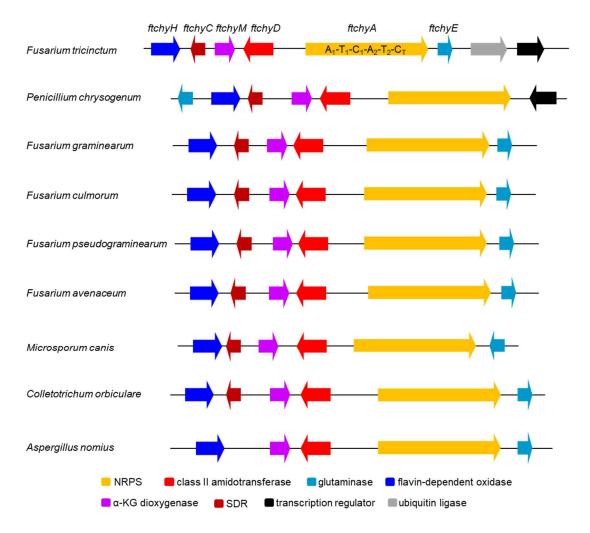


Supplementary Fig. 11 Sequence clustering analysis of the ftChyA- C_1 and ftChyA- C_T with other fungal C domains. SSN of 273 fungal C domains homologs generated by Cytoscape (v3.8.2). Each node in the network represents a protein sequence and the alignment score is 45.

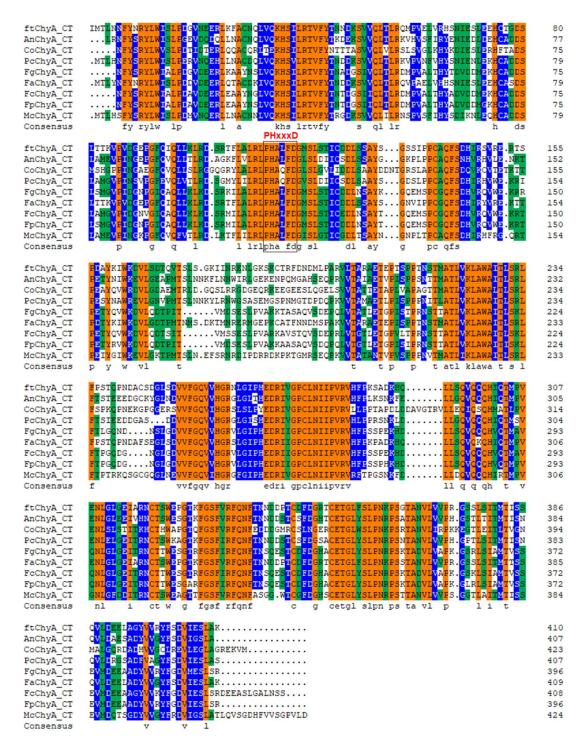
ftChyA_Cl TqaA_Cl AmpA_Cl AmpB_Cl BenZ_Cl PsyA_Cl RoqA_Cl Consensus	.EU VYECH HNQCGI DALTAQUSCAYIA SYTWFLADNVDADE FQDAW KATWINNPILESRUVQTSHGIFCVVTNTDM ENNYPOTPLQEGNVALSVSKEGYIAFFIHRIEPHVDIGSIREAREAVVQACPINETRWTDPDGSEMFOVULESF .GRAVPCSPICMGIMALSAKIFCSVARHTLDIPSVTVCEFKEVMGMIVESNTVIRTRVETDE.YGSVOVUNAPL .ED LYPCTAMQEGIMALSDSREGYVACHSLSIGPSTDINEFKERCQCWVDAHPILETCIVYPEQ.SRALCAVVQGEI .ED IFPCTPLQEGNFALGRRRCERVGEWVFEFQGMSDTEIARLTEARAAVFKEDAILETTIYPS.CRLYCVILEPP VEI IYPCTSLOEGIFALGASCCERVVGEWTYAFN.EATVDFSELRRANEVACSNALLETRIKTS.HEMVOVULNPV .ED LYPCTPLQEGIMHLSITNFGAEMGTYRFSTAPSTDINEVAMACCIWLVHILETRIQCD.GCKLOVVTRCKL .ED IFPCTPLQEGIMHLSITNFGAEMGTYRFSTAPSTDINEVAMACCIWLVHILETRIQCD.GCKLOVVTRCKL .ED IVPCTPLQEGIMHLSITNFGAEMGTYRFSTAPSTDINEVAMACCIWLVHILETRIQCD.GCKLOVVTRCKL .ED IVPCTPLQEGIMHLSITNFGAEMGTYRFSTAPSTDINEVAMACCIWLVHILETRICT .ED IVPCTPLQEGIMHLSITNFGAEMGTYRFSTAPSTDINEVAMACCIWLVHILETRICT .ED IVPCTPLQEGIMHLSITNFGAEMGTYRFSTAPSTDINEVAMACCIWLVHILETRICT .PC Q Q 1 C Q V	75 77 76 78 78 78
ftChyA_C1 TqaA_C1 AmpA_C1 AmpB_C1 BenZ_C1 PsyA_C1 RogA_C1 Consensus	FWKAVADVSGTSTEIDINCG.PIVRFFLSKASERDIHHSLFDERSIGIIACVERAYS LKDDMNSDIEVNWDMYMKPQPTDALLFGAPIVHAGILAGSDSASFESDPSASYFVVM HHCICLERWASGIMINLEKAYV VAAHDTDLAKYIELDEKIPMQIGDALAHFGIIAGFINTFVLTIHHSIYDGWSIEWIFNDVHAFE EWHTAEDLETYAKEDKARPMCAGCLITRYGLVPD.GEGWTFVWTVHHAVYDAWIFLEITNDUVHAFE EWHTAEDLETYAKEDKARPMCAGCLITRYGLVPD.GEGWTIAUTNHSVIDGWCIKLIDOVEAINH AWEDPS.RCTLEVEFGPIVMFALKKDPRICSFQIAUTNHSVIDGWCIKLIDOVEAINH FEDISGMDNCQFMNLCTFIARVTYHRGRGSSGSDSGIFLITNHALYDAWSIGULDOVEAINL g 1	133 157 141 142 148 138 141
ftChyA_C1 TqaA_C1 AmpA_C1 AmpB_C1 BenZ_C1 PsyA_C1 RoqA_C1 Consensus	GEKLEIQ.F.SF.YCHLLHQTDSSS.EDFWRQEFSELQVEHFFSASRLEQT.AEKVILEHFLHIDT.EVSTKYTLSSILR GDCUVN.SMAFSIQYLSDQLASPKTEQYWKLOFQNLAAEVFFSLPFPGYTEVPSASIHLSIPCQR.DAFGGYTMSNVIR GVVPFVRIQERDSIKEVVERNADKATEDYWRGEFAEGDMTTFFSLPSASHOFLANDSFVHTLQINR.KGPSDFISATLIR GHFLERDTIKESIQHMVTTDESES.ENFWRGEFAEGDMTTFFSLPSASHOFLANDSFVHTLQINR.KGPSDFISATLIR GHFLERDTIKESIQHMVTTDESES.ENFWRGEFAEGDMTTFFSLPSASHOFLANDSFVHTLQINR.KGPSDFISATLIR GHFLERDTIKESIQHMVTTDESES.ENFWRGEFAEGLAATREFFSAVSTSKOFVADSSVKYSMSIRTDGLSGITVASMIR RHTIPASPFSTTSVNYLRQ.LEDHKEYWTSYISCENAEVFFAILPSSYCFCPTAAATDYLDIAD.FNFGRFTRSAVIR GCSHQSPFFENREVKYISKGMSDTKCERFWRAEFAGLAADFFFSFCHHQRTFSSVHRRTINFAQ.FSPRMFTRTVAR GDKISPRPSEKHAINYISKLSIEEG.RSFWSCELKDECATMEFTSSRRFTTSPHWQVRSQCIILAE.SDMNWILANKIK W fp t	209 235 220 221 224 217 218
ftChyA_C1 TqaA_C1 AmpA_C1 AmpB_C1 BenZ_C1 PsyA_C1 RoqA_C1 Consensus	LAWAIVUNGTGEH DVVFGATVSGRNATIDGIDRLSGETLADLEVRIKLATDRFVHESLAQVONGFINMMSCETTGIS LAWAMVISCYTSEFDVVFGVTVSGRAASVADIEEMVAFILAVELRVRFSPEDTILAAFEGIONGSSEVVAFDGFGLO AAMSLVQARVCDSFETVFGCTISGRNAPVFGVEDVVAVIAUVEIKAKVDGECFVAEMIQQINSESVEMTPACNYGLO AAMGILIGSHSESIDVVFGGIVSGRNAPIANADKLFGFGLAVEVRVKFPDNDTLTIRFFGDVDQSTKWISFBCAGLO LAWAITQAQNQGNHDIVYGMTVSGRNAPVFGLAMISETVATMFFRVQLNPTASIEASUDELVECTVRGIPHECTGLO LAWAITQAQNQGNHDIVYGMTVSGRNAPVFGLAMISETVATMFFRVQLNPTASIEASUDELVECTVRGIPHECTGLO LAWAITQAQNQGNHDIVYGMTVSGRNAPVFGLAMISETVATMFFRVQLNPTASIEASUDELVECTVRGIPHECTGLO LAWAITQAQNQGNHDIVYGGTVSGRNAPVFGLAMISETVATMFFRVQLNPTASIEASUDELVECTVRGIPHECTGLO LAWAITVSSCTHENDVVFGATVSGRNAPVFEIDRIVEFFAFFFRCIEDDISVEDMVQCNCCEVSIMFFEVGIR AAVGINGUS STANDSTRAPVFGITANEVFEIDRIVEFFAFFFRCIEDDISVEDMVQCNCCEVSIMFFEVGIR AAVGINGUS STANDSTRAPVFGITANEVFEIDRIVEFFAFFFRCIEDDISVEDMVQCNCCEVSIMFFEVGIR G	287 313 298 301 302 295 296
ftChyA_C1 TqaA_C1 AmpA_C1 AmpB_C1 BenZ_C1 PsyA_C1 RogA_C1 Consensus	RIKQFGNDASEACNFCNDLVVCHHEQTTESTIFKNPVGSTSENMKAFASYFYVLICRPEKSG.VSLKMANTRDIMTPDAG RIRKVSCDADDGCSFCSCLIVOFSWAD.ENRPLINTLEAGSSVVGGASWALSVICSITDSREVNVIMEFIPNVISVPGV NIARVAESRAACNFCSDLVICFATSVAEDSIMOPFSAFQANFSTVATTLESIAVDGSTCHWHFDTVLFRLEV NIRRVSSDATAACDFCTULVVCHKEK.HLSTEEIDLRAASEADANFGTYDTLESSLKSDG.VVCSAHTDSSLVSKESV NIMKMGGEVAAACDFCTULVICTIDGTDYSLLGFPAQETSSVCOTTITULVCDLNVDF.VEFKAWYDPQLVFECV RIARWSGE XAACSFCTULLVCONER.GAGLFLLGVDEDSCNWRAFATYADTVFCDINSAF.FGLEAWTDPDIFPAQOV RIAESSSTAALGFGTULTUCONER.GAGLFLLGVDEDSCNWRAFATYADTVFCDINSAF.FGLEAWTDPDIFPAQOV RIAESSSTAALGFGTULTUCCIDTIRLQSLQ.MPPGALIELPENENHDLKFASYALSIVAQCEGTS.LGVKAIFNSCILGADRT c fq 1 f	366 392 374 379 378 372 374
ftChyA_C1 TqaA_C1 AmpA_C1 AmpB_C1 BenZ_C1 PsyA_C1 RoqA_C1 Consensus	ESLIRCISCUACQIVASEDM.LIGIVELMPPOTMAKL ERISKHFECULGILASEAG.KLSDIEIISPOTKENL GRIVRCFEFVLCQLASAFAG.KLSDIEIISPOTKENL ERIIGCIENLHQMCSSSADKKINEIFISSKIQES. RELIRCMKHVVQAICTSFAS.TVSDCMTINPSTAAEI ERIINCHAFTIEGICHGMNA.SIAT EALIECHDTLLCRILREFGT.KMKDLRTQLSFFWQCL	402 428 410 415 414 396 410

Supplementary Fig. 12 Sequences alignments of $ftChyA-C_1$ with other identified fungal NRPS C domains. The black box shows the conserved HHxxxD motif in $ftChyA-C_1$.

TqaA: ADY16697.1 (https://www.ncbi.nlm.nih.gov/protein/ADY16697.1), AmpA: A0A1W6BT53.1 (https://www.ncbi.nlm.nih.gov/protein/A0A1W6BT53.1), AmpB: A0A1W6BT46.1 (https://www.ncbi.nlm.nih.gov/protein/A0A1W6BT46.1), BenZ: P9WEU9.1 (https://www.ncbi.nlm.nih.gov/protein/P9WEU9.1), PsyA: AMQ36132.1 (https://www.ncbi.nlm.nih.gov/protein/AMQ36132.1), RoqA: B6HJU6.1 (https://www.ncbi.nlm.nih.gov/protein/B6HJU6.1).

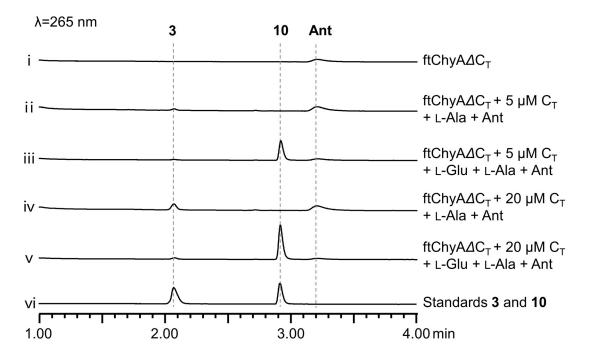


Supplementary Fig. 13 The genome-mined homologous *chy* clusters from different fungi.



Supplementary Fig. 14 Sequence alignments of ftChyA- C_T with homologous domains from other *chy* clusters. The black box shows the conserved PHxxxD motif in ftChyA- C_T .

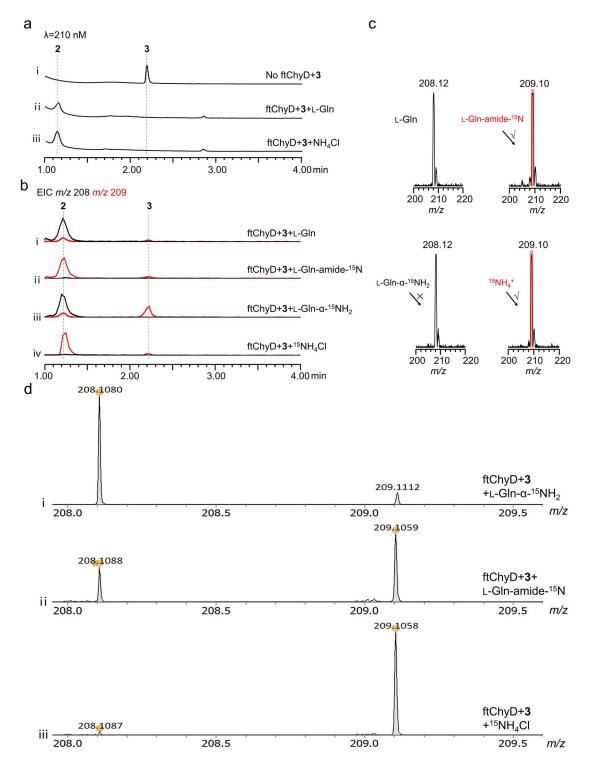
AnChyA: XP_015403503.1 (https://www.ncbi.nlm.nih.gov/protein/XP_015403503.1), CoChyA: TDZ15643.1 (https://www.ncbi.nlm.nih.gov/protein/TDZ15643.1), PcChyA: B6HLP9.1 (https://www.ncbi.nlm.nih.gov/protein/B6HLP9.1), FgChyA: I1S3K7.1 (https://www.ncbi.nlm.nih.gov/protein/I1S3K7.1), FaChyA: KIL85360.1 (https://www.ncbi.nlm.nih.gov/protein/KIL85360.1), FcChyA: QPC63060.1 (https://www.ncbi.nlm.nih.gov/protein/QPC63060.1), FpChyA: XP_009262411.1 (https://www.ncbi.nlm.nih.gov/protein/XP_009262411.1), McChyA: XP_002842771.1 (https://www.ncbi.nlm.nih.gov/protein/XP_002842771.1).



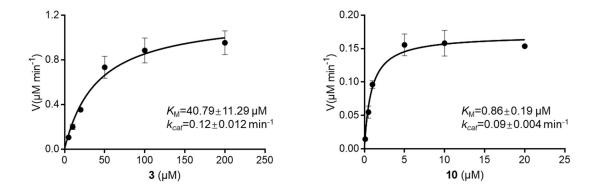
Supplementary Fig. 15 The ftChyA- C_T domain is essential for catalysing the formation of 10 and 3.

а						
Query seq.	· · · · · · · · · · · · · · · · · · ·					
dimer inte Specific hits	ards Ard					
	Aprò Apro anno 1000 Apro 1					
Non-specific asn_synth_AEB						
	lass_lactam_cya					
Superfamilies	Gn_AT_II superfamily AANH_like superfamily					
	AsnB superfamily asnB superfamily					
	asn_synth_AEB superfamily					
	lass_lactam_cya superfamily macrolact_lk_Al superfamily					
	medi o tedo "an"iti o upor tema ty					
b	c					
2	↓					
ftChyD	MCGISAFISHFGESALFVVNGEAKHVAAELESSLDIIAHRGFDARGRWFSDDYCVGLGHVRLSIIDISESGNCFFHDEEN	80				
AsnB	MCSIFGVEDIKTD	68				
Consensus	smci e ael lhrgpd g sd lhrlsid g qp					
ftChyD	GIHAV <mark>VNGELYD YERYKAOLSSEFKFVENSDCEIVIALYK</mark> HY <mark>GLSFI</mark> SHLR <mark>GEFAFVLNDAHROOLIAARDRYGI</mark> KS <mark>LY</mark> Y	160				
AsnB		148				
Consensus	s vngey a f sdce aly g f l g fafld rd gi ly					
6- 01- D		239				
ftChyD AsnB		200				
Consensus		200				
ftChyD		319				
AsnB Consensus		277				
consensu	ExxDE					
ftChyD		397				
AsnB		357				
Consensus	s a alg h d e gik v gegsdej fgg ATP binding site					
ftChyD		477				
AsnB		417				
Consensus	sy f h a g vp k l					
ftChyD	GDSIFETSIIEGFDGRVRDNIIKRWHFVHTAQYMFVKTFMEHFILEYNGDNIDMVHQVESROFFIDHHLTEYVNNIPFSL	557				
AsnB		481				
Consensus	s p g il p					
ftChyD AsnB		637 541				
Consensus		241				
ftChyD		671				
AsnB		554				
Consensus	s a					

Supplementary Fig. 16 Bioinformatic analysis of ftChyD. (**a**) Conserved domain analysis of ftChyD. (**b**) Sequence alignments of ftChyD with asparagine synthetase B (AsnB). The black arrow shows the Cys for hydrolysis. The black box shows the conserved ExxDE motif for ATP binding site. AsnB: AJE54988.1 (https://www.ncbi.nlm.nih.gov/protein/AJE54988.1).



Supplementary Fig. 17 Biochemical confirmation of the amidation of ftChyD towards 3. (a) ftChyD uses NH₄Cl or L-Gln-amide to catalyse the amidation of 3 to form 2. (b) LC-MS analysis of the incorporation of ¹⁵N into 2. The EICs were extracted at m/z 209 $[M + H]^+$ for 3 and ¹⁵N-labelled 2, and m/z 208 $[M + H]^+$ for 2. (c) Mass of 2 and ¹⁵N-labelled 2. (d) HRMS analysis of the incorporation of ¹⁵N into 2.



Supplementary Fig. 18 The kinetic parameters of ftChyD toward **3** and **10**. These values were calculated by analyzing production of **2** and **11** by LC-MS analysis. This measurement was conducted in four times. $K_{\rm M}$ and k_{cat} values represent the mean \pm SEM.

ftChyE	.MAHQTFRVAILCNFILEETSGRPMIDRLS.NLILQSKENAKITLHAPLQGDDEPDLNSH <mark>DLIILT</mark> G.GPFNLLQKEKPS	77		
PcChyE	MDSVSNIKI <mark>AVI</mark> INTPPDETDFOSVVRECFREAFASIAFTGEVDFYDPVVERKF <mark>PI</mark> ASRY <mark>DIVVI</mark> S <mark>C.</mark> CKIDAACSPP	77		
FgChyE	.MSSPSFRVAILANFILDDIGGRPMIDKIT.QLIRQSKPDALINVYAAIEGDTLPDPETK <mark>DLII</mark> I <mark>G</mark> .CPENLLKDERPK	77		
TrpG		65		
YaaE	MGSSHHHHHHSSGLVPRGSHMLTIGVLGLQGAVREHIHAIRAGAAGLVVKRPECLNEVIGLIIPG.CESTIMRRIID.	77		
HisH		60		
Consensu	as C102 g			
	↓			
ftChyE	WVTGTLDFIKAAMAGQAKSKILGICWGQCAVAIALCCSLEKSRRCHCIGVENITLNSDCALLFEAQSVNIQK	149		
PcChyE	WVLGVLDYVRITARDLPHT <mark>KILAVCWG</mark> HCAVSRAFGGQVRPVPAGEITAIEDIRLTDAGMKFFPFAATSGSYRAIE	153		
FgChyE	WVVDTLEYLKTVTAGPSKPKILGICWCCCAIAIALCCALGKSDKCQIVGIADIPLTPECTKFFESPSLTIHK	149		
TrpG	VSLEVIKYIGKETFILGVCLEHCAIGYAFGAKIRRARKVFHGKISNIILVNNSPLSIYYGIAK.EFKATR	134		
YaaE	.TYQFMEPLREFAAQGKFNFCTCAGLIILAK.EIACSINPHLGLLNVVVERNSFGRQVDSFEADITIKGLDEPFTGVF	153		
HisH	.ESGFVERVERHLERGLP <mark>FLGIC</mark> VGMCVLYEGSEEAPCVRGLGLVPGEVRRERAGRVPOMGWNALEFGGAFAPLTG	135		
Consensu				
	+ $+$			
ftChyE	N <mark>T</mark> EIAVADL GEHI SC LA P NNEILLSKDNR<mark>VLIFC</mark>G<mark>HEE</mark>MDAD<mark>I</mark>SRLEVTTDDKSPSPRPEISTDLRPIDAP	220		
PcChyE	LESGEVYTPFEGEISLAENCECFINDTNN <mark>VLTFC</mark> AHEEISHELAIGNREFTEHDS	208		
FgChyE	N <mark>H</mark> EILVIDIGEHILFLAINNEVIMSKDGQVIIFCG <mark>HFE</mark> MDSVISRLFVASDNEVIVG.AGSDSGIKFIDSP	219		
TrpG	Y <mark>H</mark> SLVVDEVHRPIIVDAISAEDNEIMAIHHEEYPIYGV <mark>OFHEE</mark> SVGTSLGYK <mark>II</mark> YNFLNRV	195		
YaaE	IRAPHILEAGENVEVISEHNGRIVAAKQQQFIGCSEHEELTEDHRVTQLEVENVEEYKQKALV	216		
HisH	R <mark>H</mark> FYFANSYYGF I TFYSLGK <mark>G</mark> EYEGTFFTALLAKEN <mark>II</mark> AF <mark>CFHEE</mark> KSGKAGLAF I ALARRYFEVL	200		
Consensu	hpe hpe			
ftChyE	HDGEAIFAVVMKWASQNEAL	240		
PcChyE		208		
FgChyE	HDGEIIFERIVRWASPETAH	239		
TrpG		195		
YaaE		216		
HisH		200		
Consensus				

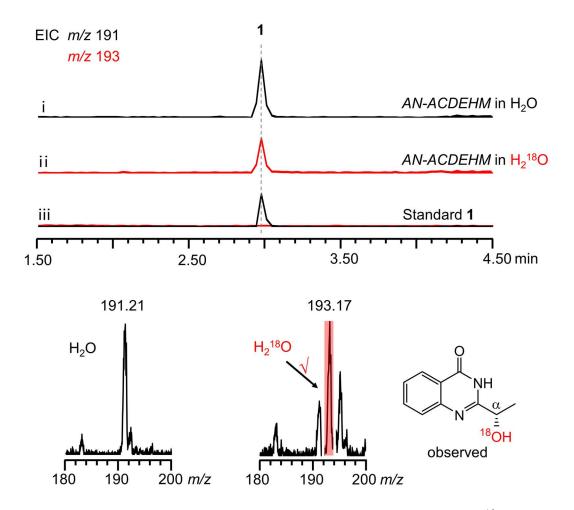
Supplementary Fig. 19 Sequence alignments of ftChyE with homologous enzymes and other identified glutaminase subunits of class I glutamine amidotransferases. The black arrow shows the catalytic triad C-H-E in ftChyE. PcChyE from *Penicillium chrysogenum*.

FgChyE: XP_011325839.1 (<u>https://www.ncbi.nlm.nih.gov/protein/XP_011325839.1</u>), TrpG: 1QDL_B (<u>https://www.ncbi.nlm.nih.gov/protein/1QDL_B</u>),

YaaE: 1R9G_A (<u>https://www.ncbi.nlm.nih.gov/protein/1R9G_A</u>),

Tade. $100^{-} \text{A} (\underline{100}, \underline{100}, \underline{100},$

HisH: 1KA9_H (<u>https://www.ncbi.nlm.nih.gov/protein/1KA9_H</u>).

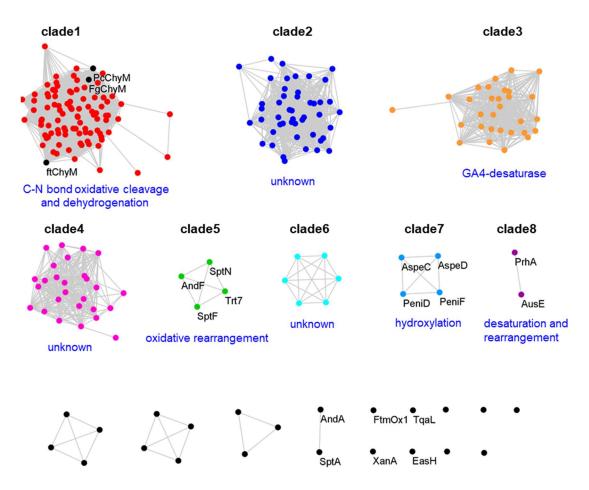


Supplementary Fig. 20 LC-MS analysis of the *AN-ftchyACDEHM* in $H_2^{18}O$ -medium showing the oxygen atom of the α -OH group of **1** could be from water. The extracted ion chromatograms (EICs) were extracted at m/z 191[M + H]⁺ for **1** and m/z 193 [M + H]⁺ for ¹⁸O-labelled **1**.

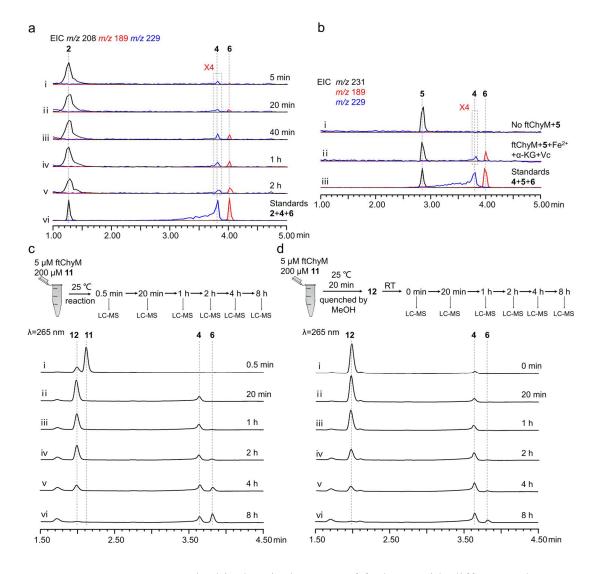
2
-

Query seq.	50	
Specific hits	FAD_binding_4	·
Non-specific	G1cD	805
hits	FAD_1actone_ox	
Superfamilies	FAD_binding_4 superfamily	BBE superfamily
	FAD_lactone_ox superfamily GlcD superfamily	L
	GICU Super+amily	
b		
ftChyM	MAATADSHLGKERYLTRGTKPTPSKEAYLLPPLSEFGDIVTLP	ITLIKPSIDIGNDSPYKISVHGFTARRHHSALHAPPY 80
GA4 desaturase		COAVAVTDIRPSVSSFTLDGNGFCVVKHTSAVGSPPY 80
Consensus	m tsapl	s l gf h sa ppy
ftChyM	DRSSWNNEKLLEDIYFFEVQSFVQNLTGOKKAVV SAAVVRNQL	YSEGDISSATETEETGOISNNEDTAELFPPIFGN 157
GA4 desaturase		FKEPELAPPYPMPGKSSSGSKEREAIPANELPTTRAK 160
Consensus	dssw ype ltgkk arn	e e a p
	HxD	
ftChyM	SVKDGICPAPKVHLDCTPKGARCHIRCYHSQVASAAGDVIE	AENSLLKSGVKWDDIKDHYCNENVSGGIPRFALFSIW 235
GA4 desaturase	GECKGEEEGEVRKEHKDWGESGAWNTLRNWSCELIDEAGDIIK	
Consensus	g p khd pga r agd i	
consensus	g p kind p ga i aga i	a x yq iai w
ftChyM	RPLRTVHRDPLALASAASFPASDYVFSDCREPKDNRIPAHLSR	IIDPNSENVONSTGONEKDEATYLTEGYLAYAPODKE 315
GA4 desaturase		SEWRNPFGVHG
Consensus	rpl tv rdp a dy d	npv t la
consensus	three controls and a	RxS
ftChyM	CRSHDWHFISRCEPSDVLVICLFDNEMEAHARAPLGEGKGVSD	
	PKHKWYWISICTEDEVILMKIMETESEKDESETAG	GVHHCSEHLPG.TEKEEVRESIETKFIAF 341
GA4_desaturase		
Consensus	hwisqpvl dee g	ghflgeeresie af

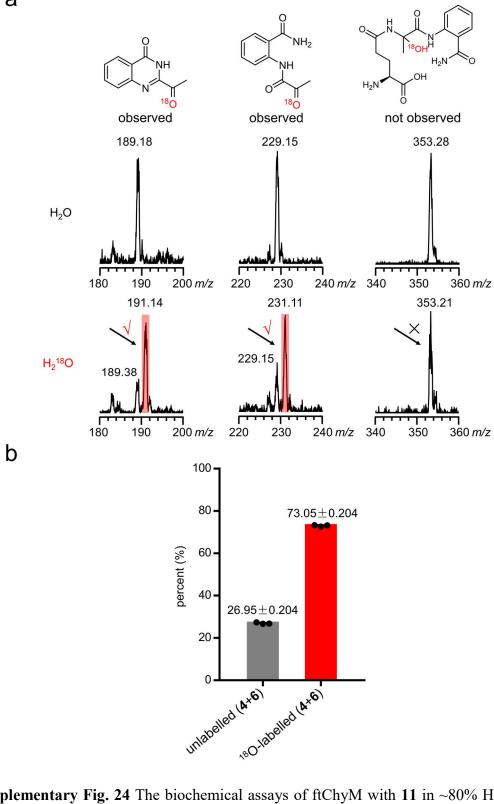
Supplementary Fig. 21 Conserved domain analysis of ftChyH and ftChyM. (**a**) ftChyH is a flavin-dependent oxidase that contains a berberine bridge enzyme (BBE) conserved domain. The red box shows that the berberine bridge enzyme (BBE) conserved domain is existed in ftChyH. (**b**) ftChyM is an α -KGD contains HxD motif and RxS motif. GA4 desaturase: CAD10289.1 (<u>https://www.ncbi.nlm.nih.gov/protein/CAD10289.1</u>). The black box shows the HxD motif for binding Fe²⁺. The blue box shows the RxS motif for binding α -ketoglutarate.



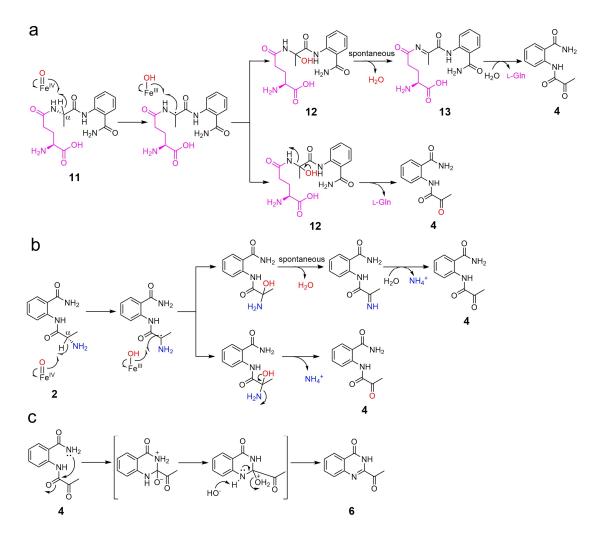
Supplementary Fig. 22 Sequence clustering analysis of ftChyM with other fungal α -ketoglutarate dependent dioxygenases. SSN of 239 fungal α -ketoglutarate dependent dioxygenases homologs generated by Cytoscape (v3.8.2). Each node in the network represents a protein sequence and the alignment score is 60.



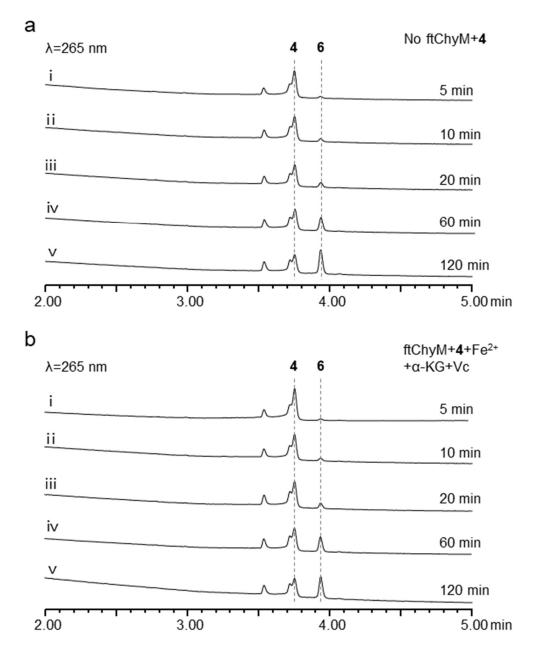
Supplementary Fig. 23 The biochemical assays of ftChyM with different substrates. (a) Time course assays of ftChyM with 2. (b) ftChyM catalyse dehydrogenation of 5 to form 4. (c) Time course assays of ftChyM with 11. (d) Time course assays of *in situ* synthesized 12 spontaneously converted to 4 and 6 in room temperature.



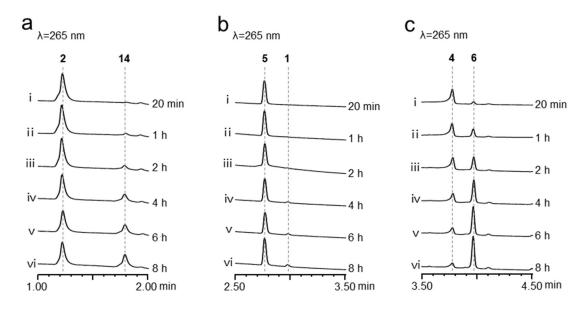
Supplementary Fig. 24 The biochemical assays of ftChyM with 11 in ~80% $H_2^{18}O$ -Tris-HCl buffer. (a) LC-MS analysis of the ftChyM with 11 in $H_2^{18}O$ show that ¹⁸O was incorporated into 4 and 6. (b) Analysis of unlabeled and ¹⁸O-labelled 4 and 6 (in sum). Data was shown as mean ± SEM for 3 independent experiments.



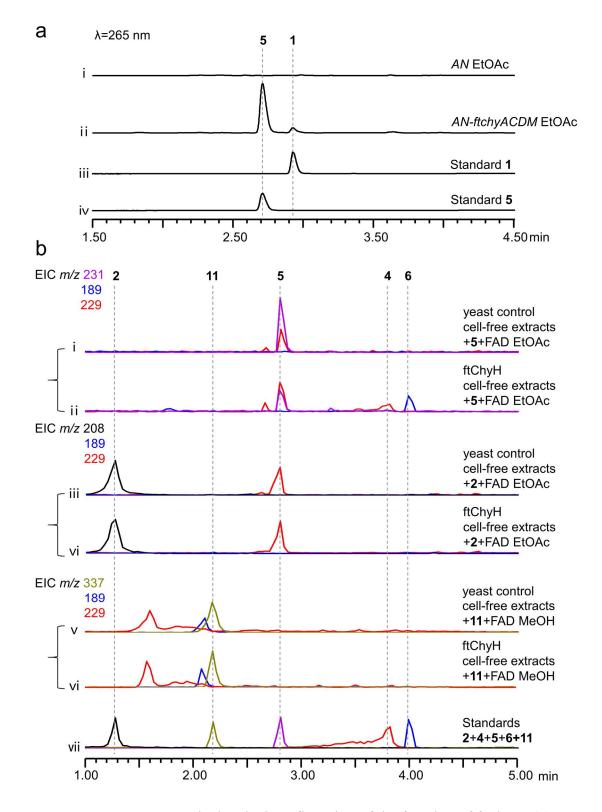
Supplementary Fig. 25 The proposed mechanism of the (**a**) ftChyM-catalyzed C-N bond oxidative cleavage **11** to form **4**; (**b**) the ftChyM-catalyzed oxidative deamination of **2** to form **4**; (**c**) the alkaline-induced spontaneous C-2-N-3 bond closure of **4** to form **6**.



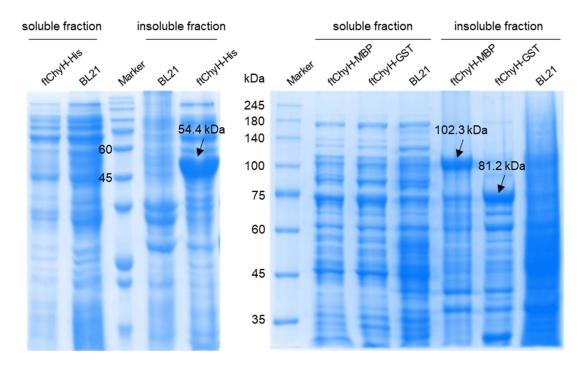
Supplementary Fig. 26 Time-course assays of the spontaneous conversion of 4 to 6.(a) Tris-HCl buffer (pH 7.5); (b) with ftChyM addition.



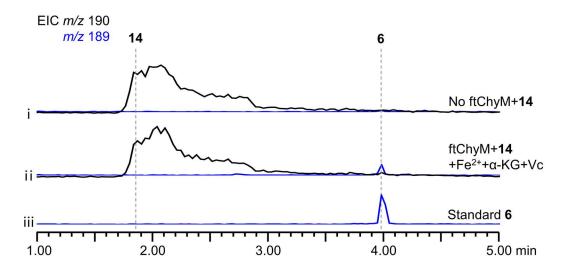
Supplementary Fig. 27 Spontaneous cyclization of compound **2**, **5** and **4** in Tris-HCl buffer (pH 7.5). (a) cyclization of **2** to form **14**; (b) cyclization of **5** to form **1**; (c) cyclization of **4** to form **6**.



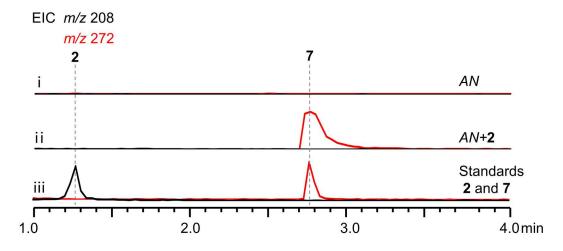
Supplementary Fig. 28 Biochemical confirmation of the function of ftChyH. (a) LC-MS analyses of the *AN-ftchyACDM* showing elimination of *ftchyH* accumulates **5**. (b) Biochemical assay of ftChyH cell-free extracts with **5**, **2** and **11**, respectively. The extracted ion chromatograms (EICs) were extracted at m/z 208 [M + H]⁺ for **2**, m/z 229 [M + Na]⁺ for **4**, m/z 231 [M + Na]⁺ for **5**, m/z 189 [M + H]⁺ for **6**, m/z 337 [M + H]⁺ for **11**.



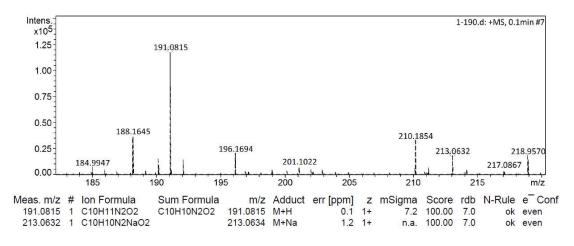
Supplementary Fig. 29 SDS-PAGE analyses of the expression of ftChyH-His, ftChyH-MBP and ftChyH-GST in *E. coli* BL21, respectively. All experiments were repeated independently more than three times with similar results.



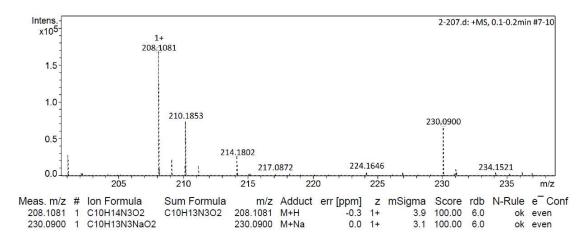
Supplementary Fig. 30 Biochemical assay of ftChyM with **14** *in vitro*. The extracted ion chromatograms (EICs) were extracted at m/z 190 [M + H]⁺ for **14** and m/z 189 [M + H]⁺ for **6**.



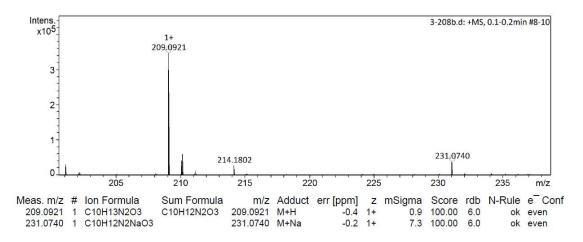
Supplementary Fig. 31 LC-MS analyses of the *A. nidulans* with feeding **2** showing that **2** was converted to **7** by an unknown acetyltransferase of *A. nidulans*. The extracted ion chromatograms (EICs) were extracted at m/z 208 [M + H]⁺ for **2** and m/z 272 [M + Na]⁺ for **7**.



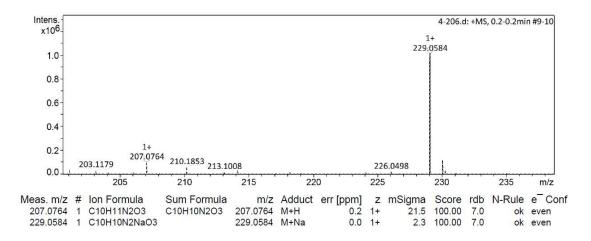
Supplementary Fig. 32 HRMS spectrum (positive ionization) of compound 1.



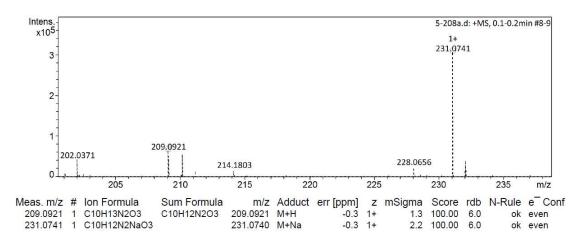
Supplementary Fig. 33 HRMS spectrum (positive ionization) of compound 2.



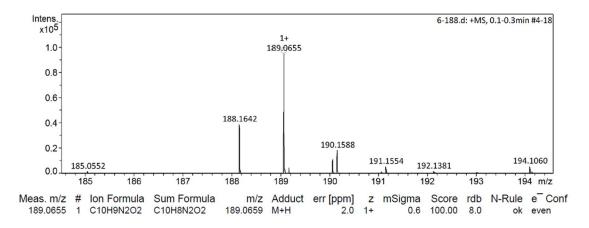
Supplementary Fig. 34 HRMS spectrum (positive ionization) of compound 3.



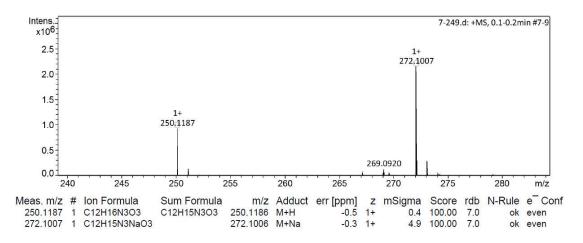
Supplementary Fig. 35 HRMS spectrum (positive ionization) of compound 4.



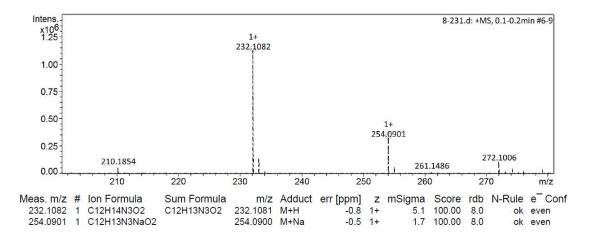
Supplementary Fig. 36 HRMS spectrum (positive ionization) of compound 5.



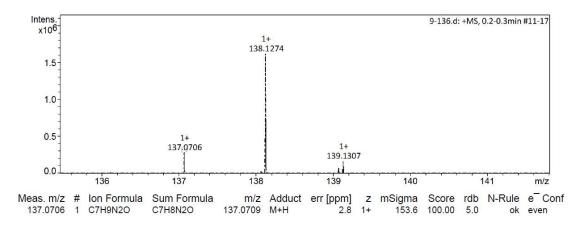
Supplementary Fig. 37 HRMS spectrum (positive ionization) of compound 6.



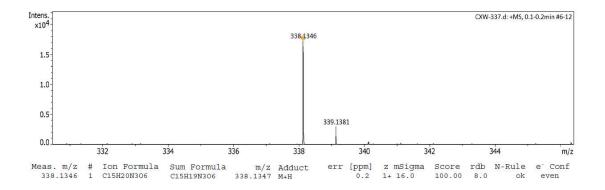
Supplementary Fig. 38 HRMS spectrum (positive ionization) of compound 7.



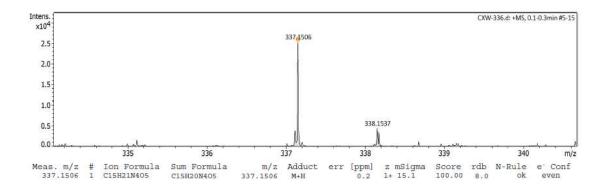
Supplementary Fig. 39 HRMS spectrum (positive ionization) of compound 8.



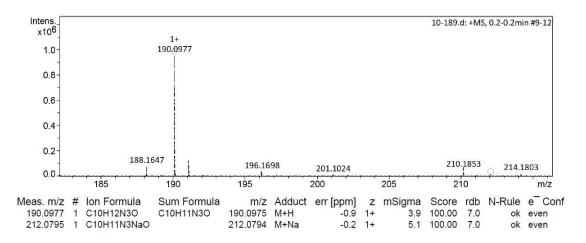
Supplementary Fig. 40 HRMS spectrum (positive ionization) of compound 9.



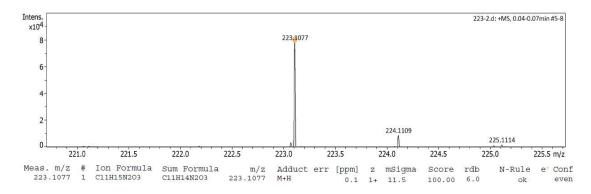
Supplementary Fig. 41 HRMS spectrum (positive ionization) of compound 10.



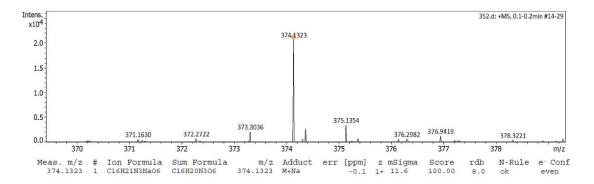
Supplementary Fig. 42 HRMS spectrum (positive ionization) of compound 11.



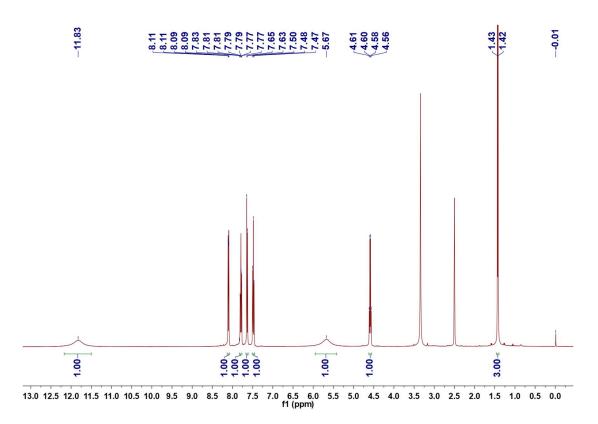
Supplementary Fig. 43 HRMS spectrum (positive ionization) of compound 14.



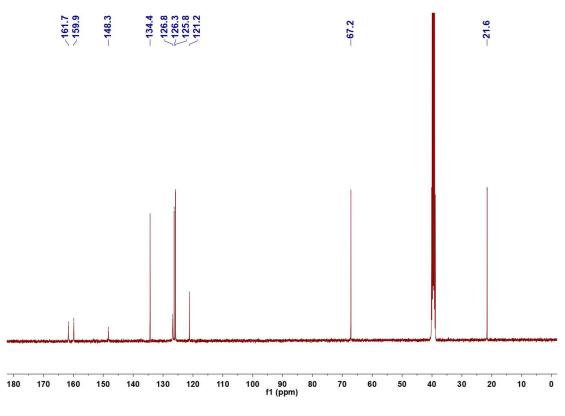
Supplementary Fig. 44 HRMS spectrum (positive ionization) of compound Ant-Me-3.



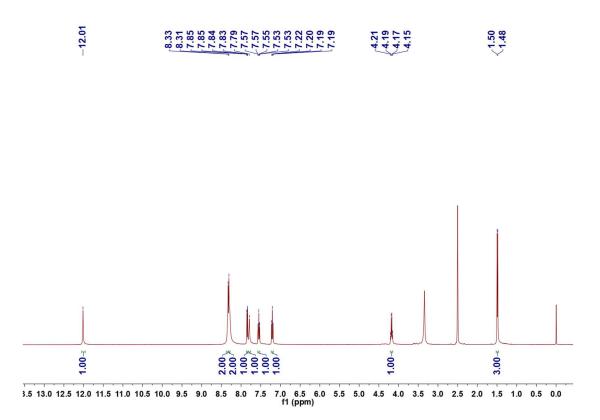
Supplementary Fig. 45 HRMS spectrum (positive ionization) of compound Ant-Me-10.



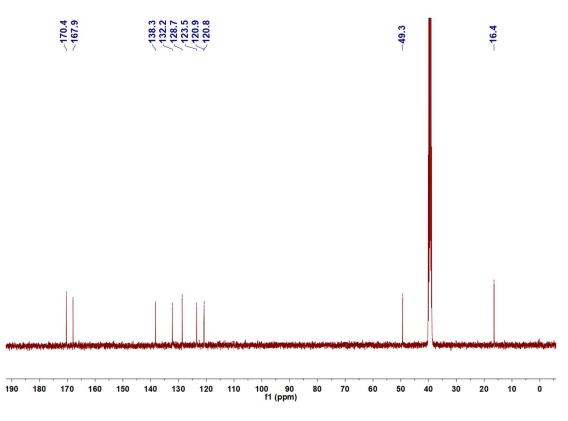
Supplementary Fig. 46 ¹H NMR spectrum of compound 1 in DMSO- d_6 (400 MHz).



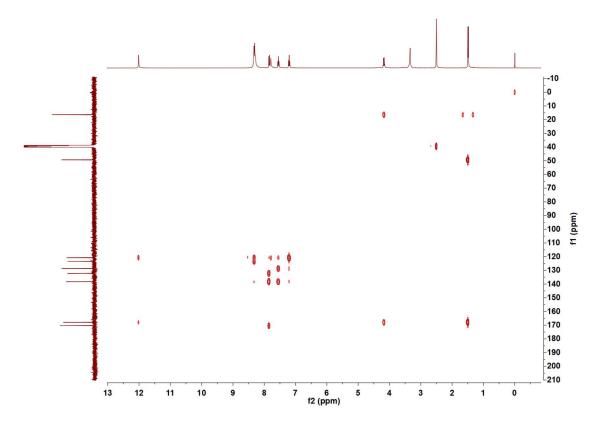
Supplementary Fig. 47 ¹³C NMR spectrum of compound 1 in DMSO- d_6 (100 MHz).



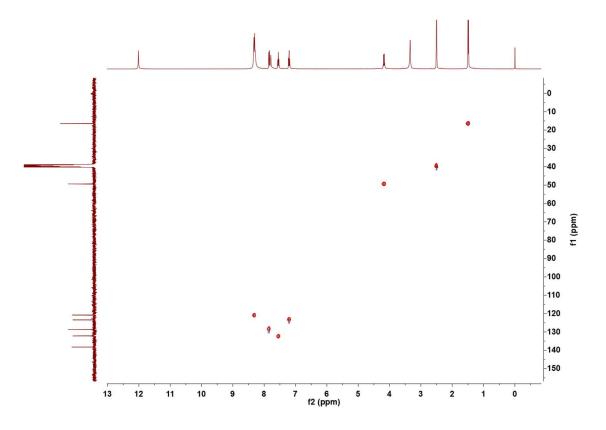
Supplementary Fig. 48 ¹H NMR spectrum of compound 2 in DMSO-*d*₆ (400 MHz).



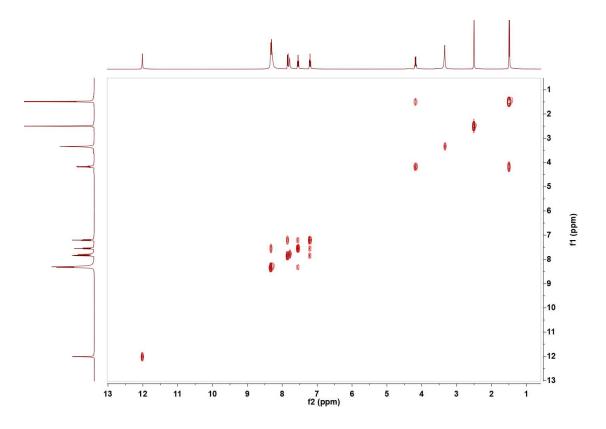
Supplementary Fig. 49 ¹³C NMR spectrum of compound 2 in DMSO- d_6 (100 MHz).



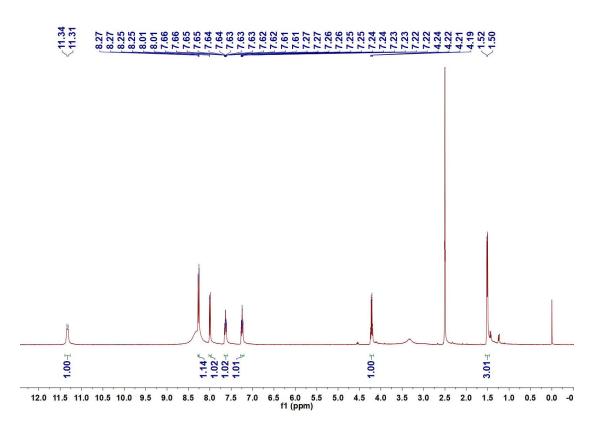
Supplementary Fig. 50 HMBC spectrum of compound 2 in DMSO-d₆.



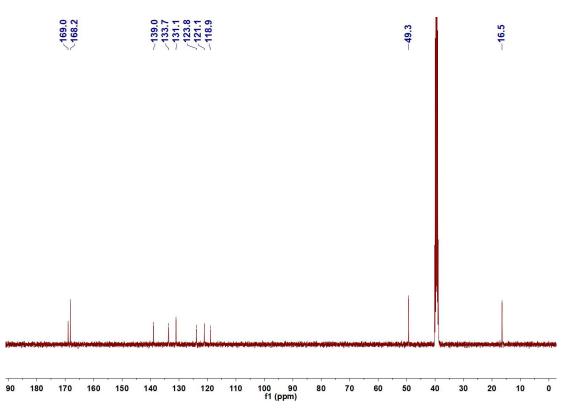
Supplementary Fig. 51 HSQC spectrum of compound 2 in DMSO- d_6 .



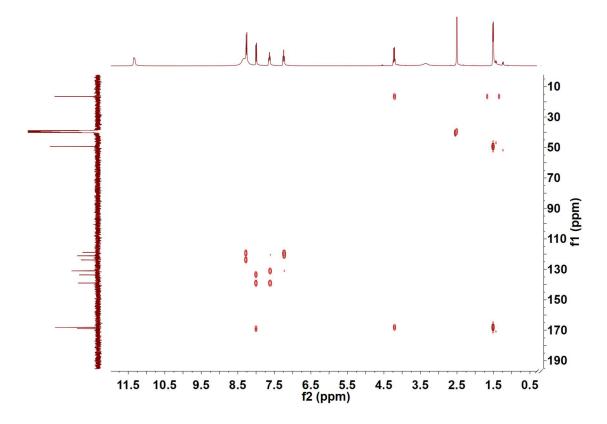
Supplementary Fig. 52 1 H- 1 H-COSY spectrum of compound 2 in DMSO- d_{6} .



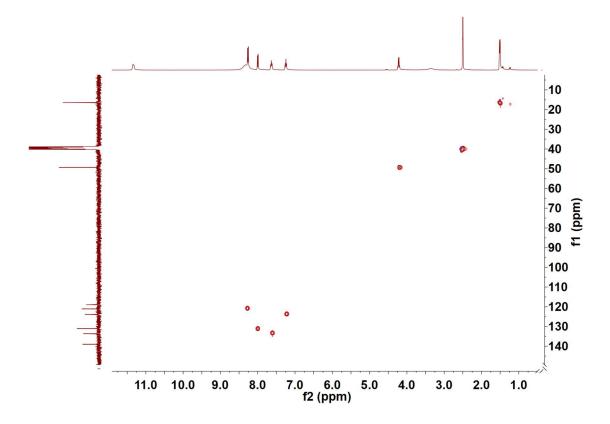
Supplementary Fig. 53 ¹H NMR spectrum of compound 3 in DMSO-*d*₆ (400 MHz).



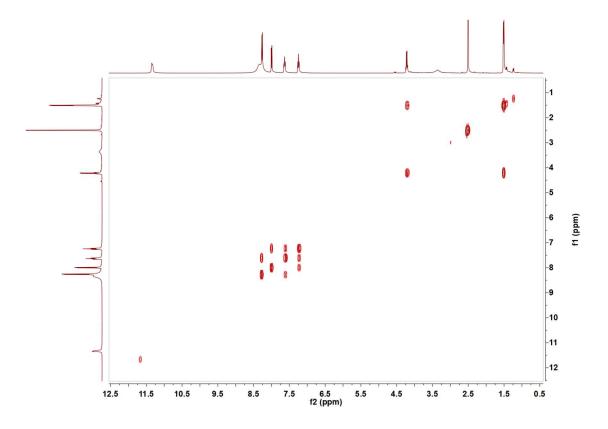
Supplementary Fig. 54 13 C NMR spectrum of compound 3 in DMSO- d_6 (100 MHz).



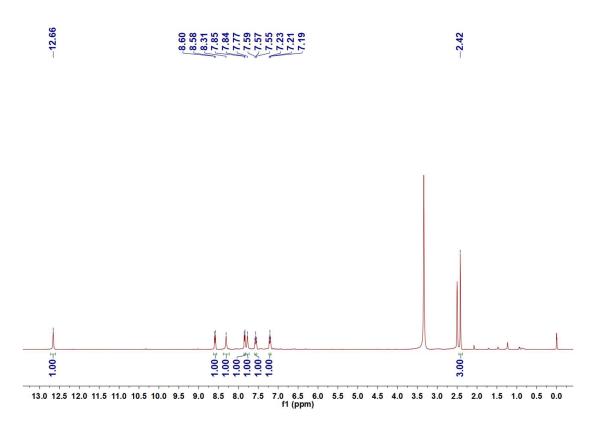
Supplementary Fig. 55 HMBC spectrum of compound 3 in DMSO- d_6 .



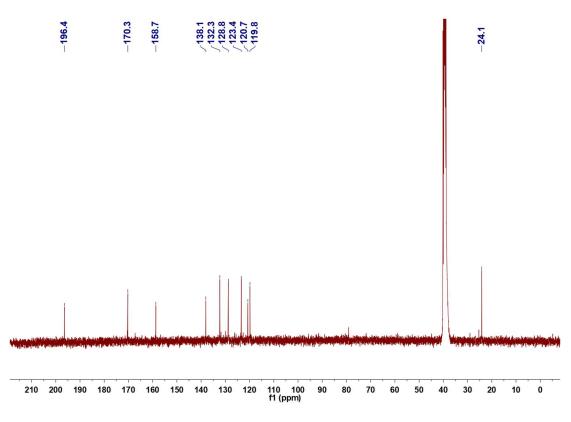
Supplementary Fig. 56 HSQC spectrum of compound 3 in DMSO-d₆.



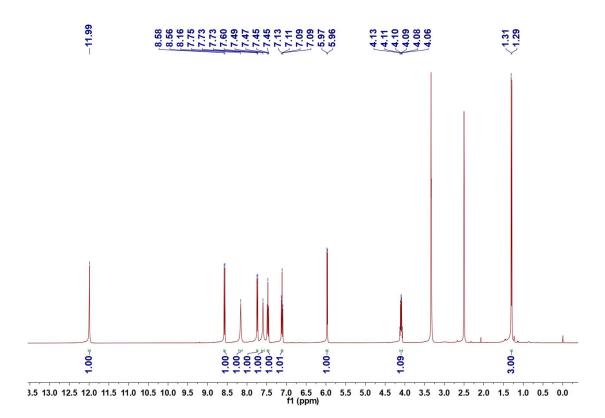
Supplementary Fig. 57 1 H- 1 H-COSY spectrum of compound 3 in DMSO- d_{6} .



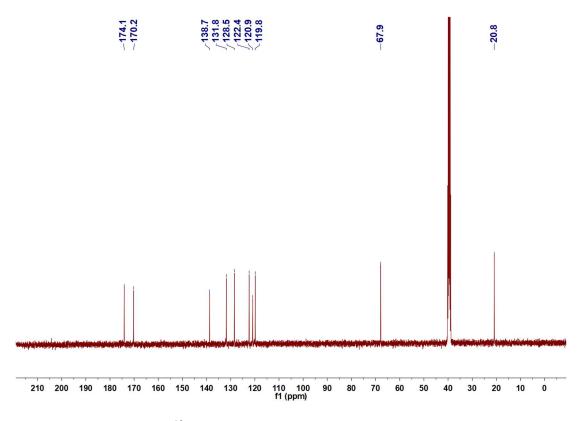
Supplementary Fig. 58 ¹H NMR spectrum of compound 4 in DMSO- d_6 (400 MHz).



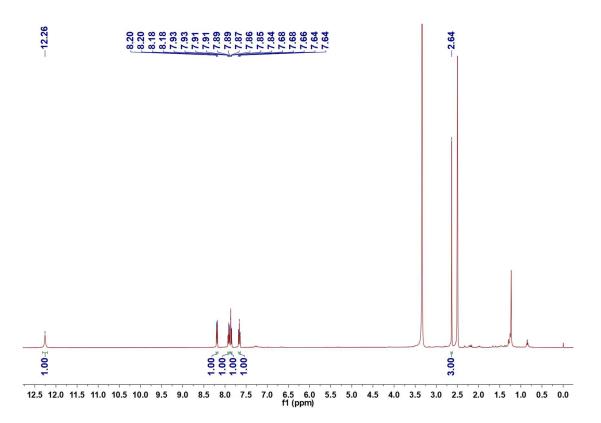
Supplementary Fig. 59 ¹³C NMR spectrum of compound 4 in DMSO- d_6 (100 MHz).



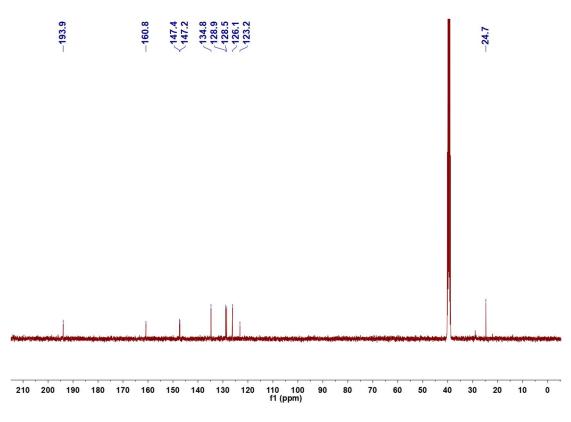
Supplementary Fig. 60 ¹H NMR spectrum of compound 5 in DMSO- d_6 (400 MHz).



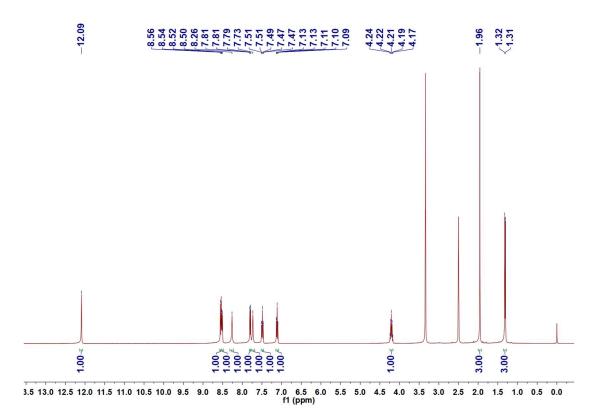
Supplementary Fig. 61 ¹³C NMR spectrum of compound 5 in DMSO- d_6 (100 MHz).



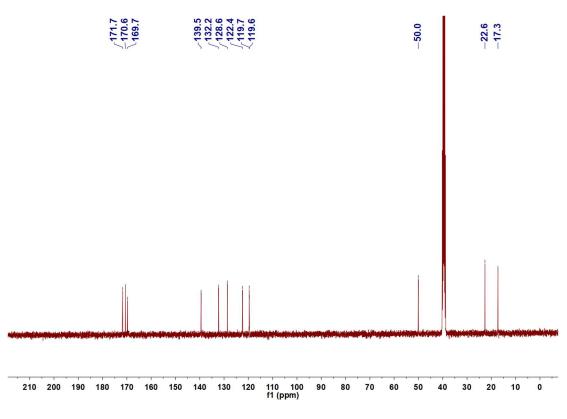
Supplementary Fig. 62 ¹H NMR spectrum of compound 6 in DMSO- d_6 (400 MHz).



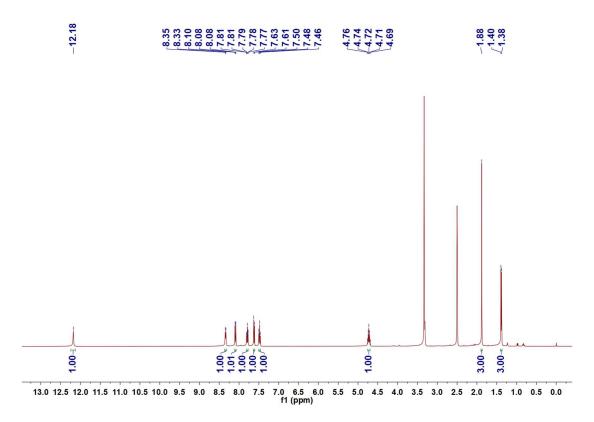
Supplementary Fig. 63 ¹³C NMR spectrum of compound 6 in DMSO- d_6 (100 MHz).



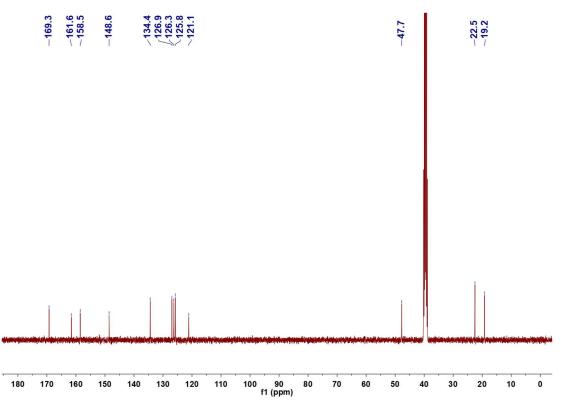
Supplementary Fig. 64 ¹H NMR spectrum of compound 7 in DMSO- d_6 (400 MHz).



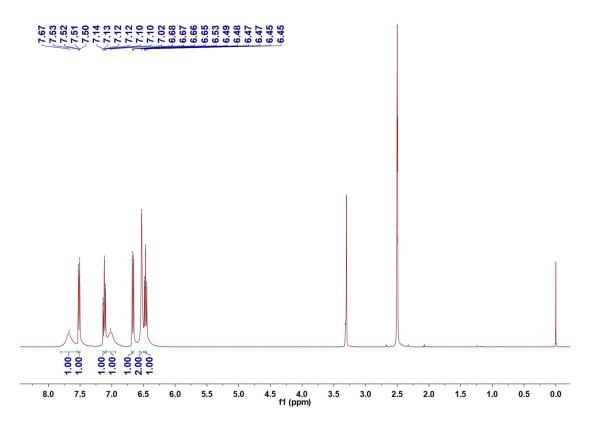
Supplementary Fig. 65 ¹³C NMR spectrum of compound 7 in DMSO- d_6 (100 MHz).



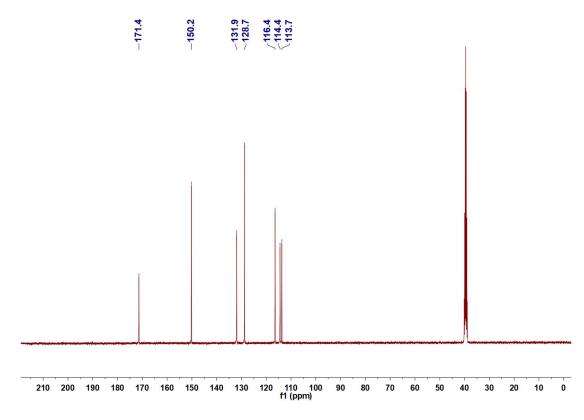
Supplementary Fig. 66 ¹H NMR spectrum of compound 8 in DMSO- d_6 (400 MHz).



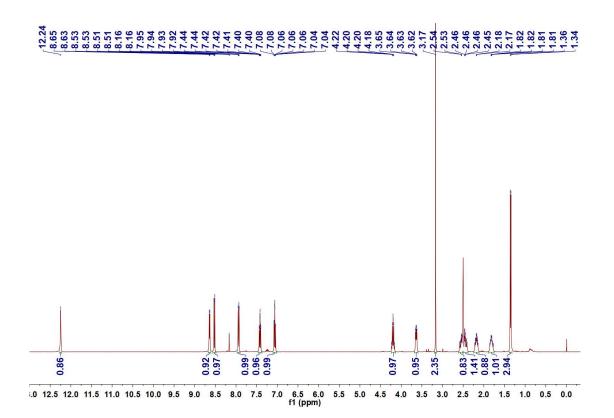
Supplementary Fig. 67 ¹³C NMR spectrum of compound 8 in DMSO- d_6 (100 MHz).



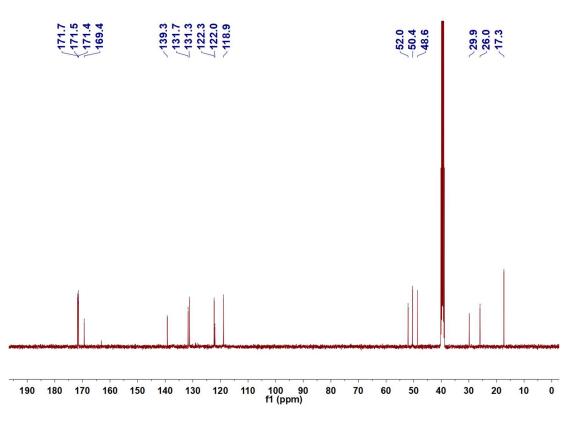
Supplementary Fig. 68 ¹H NMR spectrum of compound 9 in DMSO- d_6 (400 MHz).



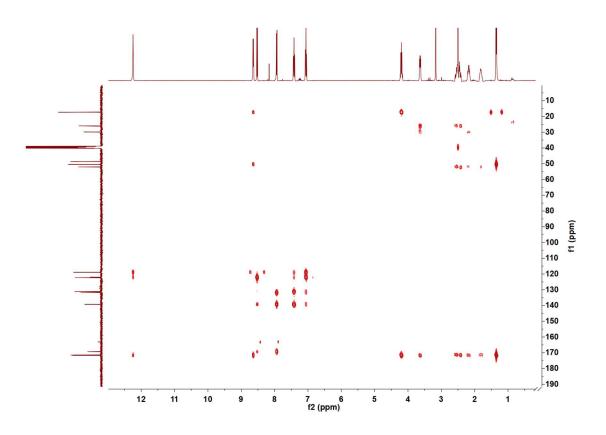
Supplementary Fig. 69 ¹³C NMR spectrum of compound 9 in DMSO- d_6 (100 MHz).



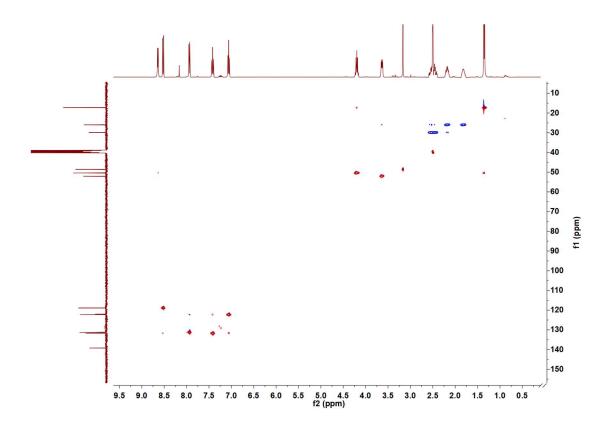
Supplementary Fig. 70 ¹H NMR spectrum of compound 10 in DMSO- d_6 (400 MHz).



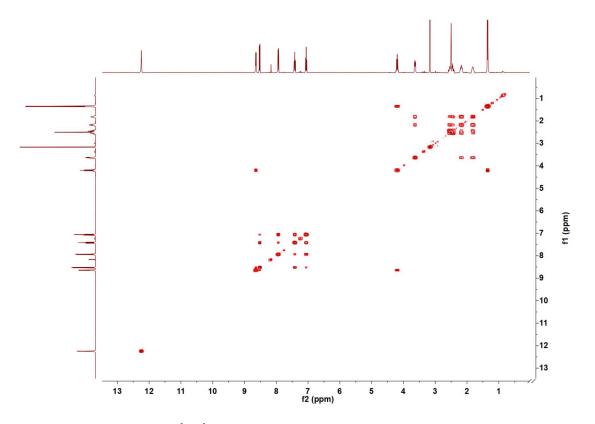
Supplementary Fig. 71 ¹³C NMR spectrum of compound 10 in DMSO- $d_6(100 \text{ MHz})$.



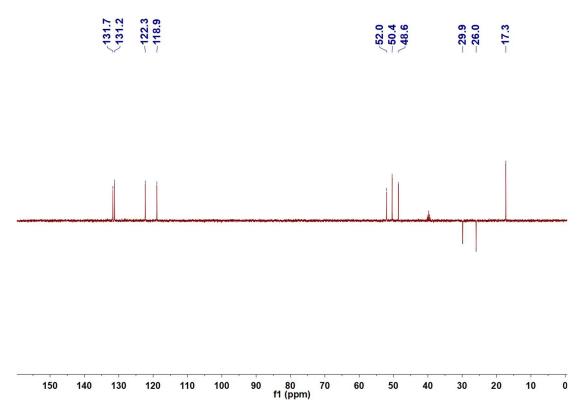
Supplementary Fig. 72 HMBC spectrum of compound 10 in DMSO-d₆.



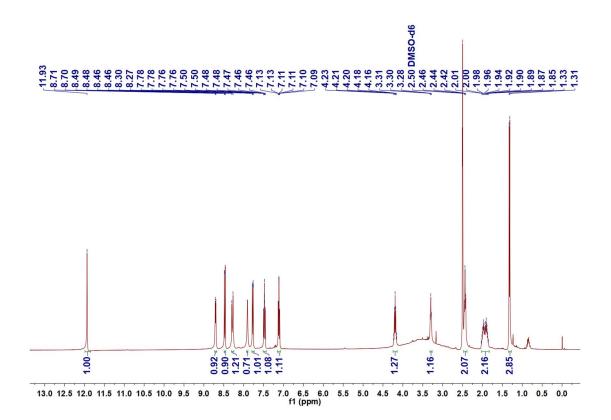
Supplementary Fig. 73 HSQC spectrum of compound 10 in DMSO-d₆.



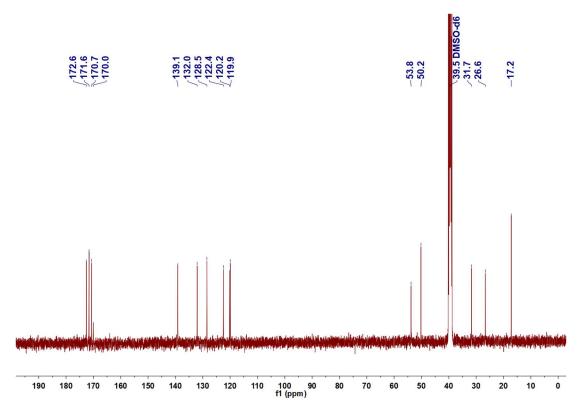
Supplementary Fig. 74 ¹H-¹H COSY spectrum of compound 10 in DMSO-d₆.



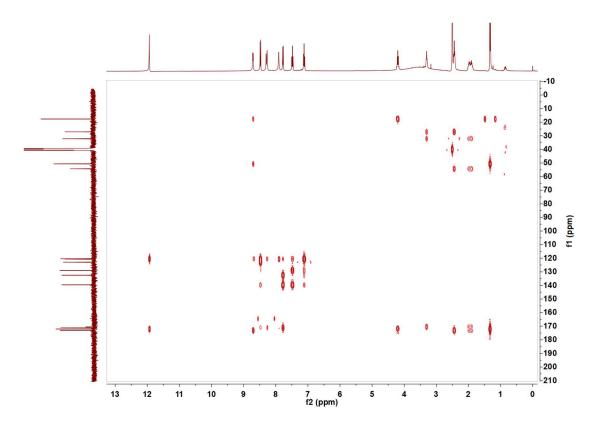
Supplementary Fig. 75 DEPT-135° spectrum of compound 10 in DMSO-d₆.



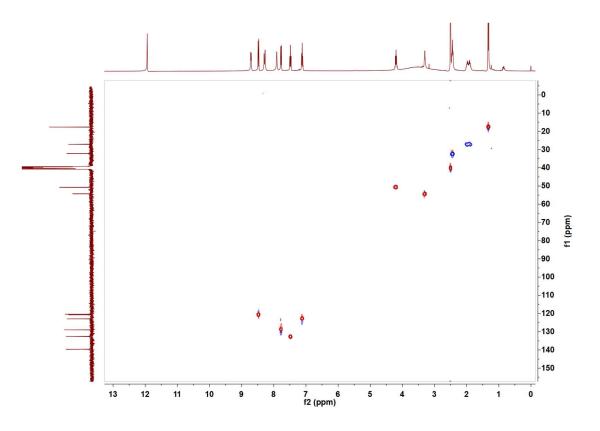
Supplementary Fig. 76 ¹H NMR spectrum of compound 11 in DMSO- d_6 (400 MHz).



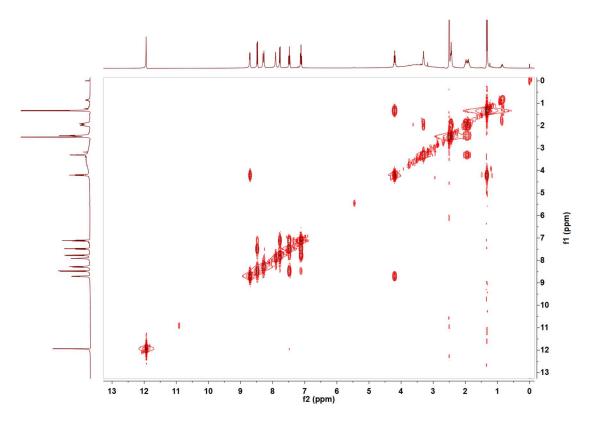
Supplementary Fig. 77 ¹³C NMR spectrum of compound 11 in DMSO- $d_6(100 \text{ MHz})$.



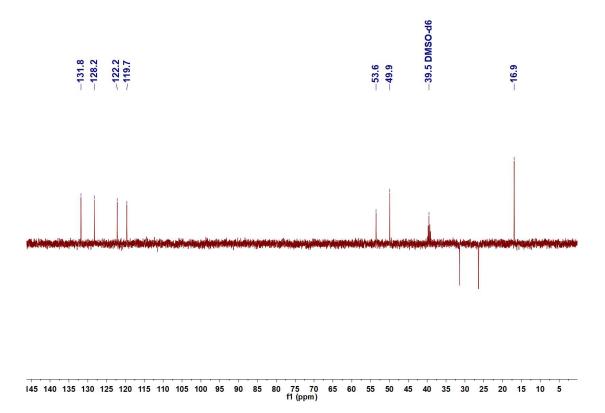
Supplementary Fig. 78 HMBC spectrum of compound 11 in DMSO-d₆.



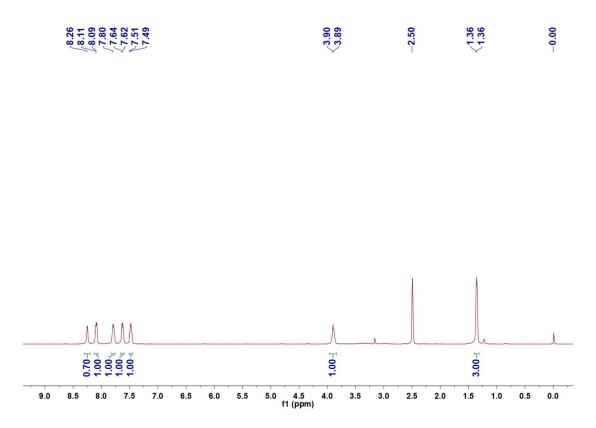
Supplementary Fig. 79 HSQC spectrum of compound 11 in DMSO-d₆.



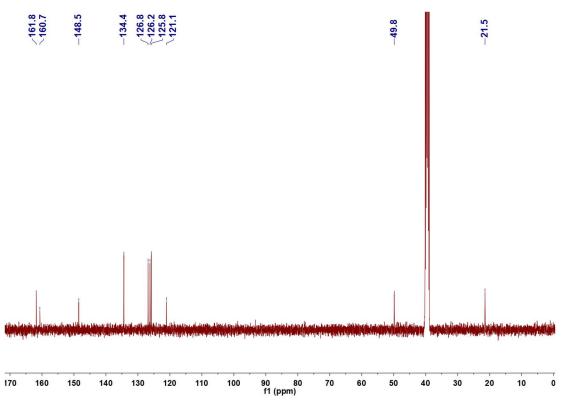
Supplementary Fig. 80 ¹H-¹H COSY spectrum of compound 11 in DMSO-d₆.



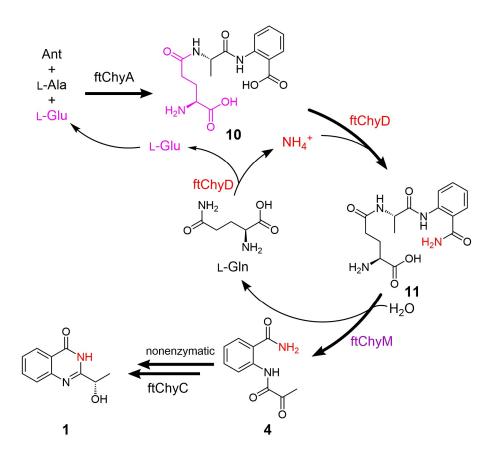
Supplementary Fig. 81 DEPT-135° spectrum of compound 11 in DMSO-d6.



Supplementary Fig. 82 ¹H NMR spectrum of compound 14 in DMSO- d_6 (400 MHz).



Supplementary Fig. 83 13 C NMR spectrum of compound 14 in DMSO- $d_6(100 \text{ MHz})$.



Supplementary Fig. 84 An efficient self-circulation system among ftChyA, ftChyD and ftChyM-catalysed reactions.

4. Supplementary References

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