Electronic Supplementary Information

New insights into the disulfide bond formation enzymes in epidithiodiketopiperazine alkaloids

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Contents

Experimental section

Strains, media and growth conditions

The fungal strains used in this study are summarized in **Table S1**. *T. hypoxylon* was isolated from the stroma of *Hypoxylon anthochroum* in Thailand and deposited in the China General Microbiological Culture Collection Center (CGMCC 3.17906, Beijing, China).[1] The wild type (WT) strain was usually cultivated on potato dextrose (BD) agar (PDA) at 25°C. For detection of secondary metabolites (SMs), the WT strain was cultivated on rice medium. *E. coli* DH5α and BL21(DE3) were grown in LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) for standard DNA manipulation. 100 μg mL^{-1} ampicillin or 50 μg mL^{-1} kanamycin were supplemented for cultivation of recombinant *E. coli* strains.

Computer-assisted sequence analysis

Protein sequences used in this study were taken from the NCBI database (http://www.ncbi.nlm.nih.gov/protein) and compared with each other by using BLASTP program (http://blast.ncbi.nlm.nih.gov/). Cluster analysis of TdaR homologs was carried out by blast using an EFI Enzyme Similarity Tool (https://efi.igb.illinois.edu/efi-est) and Cytoscape (http://www.cytoscape.org/). We cut off the primary results by filtered for 80% maximum sequence identity for analysis. Multiple sequence alignments were carried out with the program ClustalW and visualized with ESPript 3.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) to

identify strictly conserved amino acid residues.[2]

Genomic DNA isolation

The mycelia of *T. hypoxylon* were collected in 1.5 mL tubes by using miracloth. Four steel beads (about 3 mm in diameter) and 400 μL of LETS buffer (10 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 0.5% SDS and 0.1 M LiCl) were added to the tubes. After vigorous mixing for 30 s, 300 μL LETS buffer was added, and the solution was then treated with 700 μL phenol: chloroform: isoamyl alcohol (25: 24: 1). The genomic DNA was precipitated by addition of 900 μL absolute ethanol. After centrifugation at 13,000 rpm for 30 min and washing with 70% ethanol, the obtained DNA was dissolved in 50 μL distillated H_2O.

RNA isolation and cDNA synthesis

T. hypoxylon strain was cultivated on rice media containing 60 g rice and 90 mL water at 25°C for 7 d. *Aspergillus oryzae* and *Aspergillus fumigatus* were cultivated in LMM media (1.0% glucose, 50 mL L^{-1} salt solution, 1 mL L^{-1} trace element solution and 0.5% yeast extract) for 4 days. RNA extraction was performed by using TRIZOL reagent (Invitrogen, USA) according to the manufacturer´s instruction. The FastQuant RT Kit (TIANGEN) was used for cDNA synthesis with Oligo-dT primers.

Construction of deletion cassettes and genetic manipulation

The oligonucleotide sequences for PCR amplification are given in **Table S2**. For creation of *tdaA* and *tdaR* deletion strains, approximately 1.6 kbp sequences located

upstream and downstream the target genes were amplified from genomic DNA of *T. hypoxylon* using primer pairs *tda*-QC-5F F/R and *tda*-QC-3F F/R **(Table S2)**. These two fragments were subsequently inserted into pUCH2-8 containing a hygromycin (*hph*) resistance gene to construct the plasmid pYHL14 and pYHL39 using the Quick-Change method described previously.[3] Transformation was performed using PEG-mediated protoplast transformation described in a previous study.[4,5] Transformants (TYHL4 and TYHL23) were verified using three pairs of designed primers *tda*-SCR F/*hph*-SCR R, *hph*-SCR F/*tda*-SCR R as well as *tdaA*-RT F/R, and selected twice using hygromycin to obtain mitotic stability **(Table S2)**.

Overproduction and purification of TdaR, AclT and GliT in *E. coli*

The coding DNA of TdaR was amplified using primer pairs tdaR QC F/tdaR QC R. The DNA fragment was inserted into pET28a(+)SUMO vector by Quick-Change to obtain the overexpression plasmid pYHL79, which was confirmed by sequencing. *E. coli* BL21(DE3) cells harboring the plasmid pYHL79 were cultivated in a 1000 mL Erlenmeyer flask containing 300 mL liquid LB medium supplementvropriate antibiotics and grown at 37°C to an absorption of 0.6 at 600 nm OD. Isopropyl thiogalactoside (IPTG) was added to a final concentration of 0.5 mM and the cells were cultivated for further 16 h at 28°C for induction. Pellets were collected by centrifuging, resuspended in lysis buffer (10 mM imidazole, 50 mM NaH_2PO4 and 300 mM NaCl, pH 8.0) and lysed on ice by sonication for 20 minutes. The lysate was centrifuged at 15, 000 g for 60 min at 4°C to remove the cellular debris. One-step purification of the

recombinant His_6-tagged fusion protein by affinity chromatography with Ni-NTA agarose resin was carried out according to the manufacturer's instructions. In order to change the buffer, the purified protein was passed through a PD-10 column, which had been equilibrated with 50 mM Tris-HCl, pH 7.5 previously. The purified protein was eluted with the same buffer containing 20% glycerol before storage at -80°C. The purified enzyme was checked by SDS-PAGE **(Figure 4)**. Bradford Protein Assay (Bio-Rad) was used to calculate protein concentration. The same method was used to purify the proteins of AclT and GliT.

Preparation of red-pretrichodermamide A and red-gliotoxin

To carry out enzyme assays of TdaR, AclT and GliT, 1 mM of pretrichodermamide A (**1**) and gliotoxin (**6**) (Biopurify phytochemicals Ltd.), dissolved in degassed acetonitrile, were reduced using 1 mM of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as reducing agent in a total volume of 500 μL phosphate buffer (0.1 M, pH 6.5), respectively. After extracted with ethyl acetate, the products were purified on semi-preparative HPLC. Red-pretrichodermamide A (**5**) was eluted after 13.5 min (acetonitrile/H_2O, 35:65). Red-gliotoxin (**6**) was eluted after 12 min (acetonitrile/H_2O, 18:82).

***In vitro* assays of TdaR, AclT and GliT**

To determine the enzyme activity of TdaR, AclT and GliT toward **4**, incubation of 0.1 μM enzyme with 0.5 mM of pure substrate **4** was performed in phosphate buffer (pH 6.5, 0.1 M) at 37°C to a total volume of 100 μL. The reaction was terminated after 30

min with trifluoroacetic acid (TFA, 2%), filtered and measured with HPLC (method A). As a negative control, the same experiment was carried out with heat-inactivated enzymes. **4** was eluted after 9.8 min, and **1** eluted after 10.4 min.

To determine the enzyme activity of TdaR, AclT and GliT toward **5**, incubation of 0.1 μM enzyme with 0.5 mM of pure substrate **5** was performed in phosphate buffer (pH 6.5, 0.1 M) at 37°C to a total volume of 100 μL. The reaction was terminated after 30 min with trifluoroacetic acid (TFA, 2%), filtered and measured with HPLC (method A). As a negative control, the same experiment was carried out with heat-inactivated enzymes. **5** eluted after 5.8 min, and **6** eluted after 7.3 min.

Since **4** and **5** could be transformed to **1** and **6** spontaneously in the negative control, we further carried out the enzyme assays with a mixture containing **1** and DTT as substrate. In detail, 0.5 mM of **1** was incubated with and 1 mM of DTT as reducing agent in a total volume of 50 μL phosphate buffer (0.1 M, pH 6.5). The reaction was completed after 30 min at 30°C to convert all **1** to **4** (mixture A). To determine the enzyme activity of TdaR, AclT and GliT toward **4**, 0.1 μM enzyme in phosphate buffer (pH 6.5, 0.1 M) was directly added into mixture A at 37°C to a total volume of 100 μL. The reaction was terminated after 5, 10, 15, 20 and 30 min with trifluoroacetic acid (TFA, 2%), filtered and measured with HPLC (method A). Determination of TdaR activity without O_2 was carried out in a O_2-free glove box, in which N_2 was circulated.

6 was reduced to **5** in a similar way to **1**. After incubation at 30°C after 30 min, **6** was completely transformed to **5** (mixture B). To determine the enzyme activity of TdaR, AclT and GliT toward **5**, 0.1 μM enzyme in phosphate buffer (0.1 M, pH 6.5) was

directly added into mixture B at 37°C to a total volume of 100 µL. The reaction was terminated after 5, 10, 15, 20 and 30 min with TFA (2%), filtered and measured with HPLC (method B).

Large-scale fermentation and isolation of pretrichodermamide A (1)

To isolate **1**, *T. hypoxylon* WT strain was cultivated in 50 x 500 mL flasks each containing 60 g rice and 90 mL H_2O at 25°C for 14 days. The rice cultures were extracted with 5 L ethyl acetate and concentrated under reduced pressure to obtain a crude extract (8.9 g). The crude extract was applied to MCI gel reversed phase chromatography column and eluted with a stepwise gradient acetonitrile/H_2O (5:95, 10:90, 30:70, 50:50, 70:30, 90:10 and 100:0), yielding 7 fractions. Fraction 2 was purified on a semi-preparative HPLC (acetonitrile/H_2O, 20:80) yielding compound **1** (3 mg).

HPLC and LC-MS analysis

HPLC analysis was conducted with a Waters HPLC system (Waters e2695, Waters 2998, Photodiode Array Detector) using an ODS column (C18, 250_4.6 mm, aters Pak, 5 µm). Water with 0.1% (*v/v*) formic acid (A) and acetonitrile (B) were used as solvents at the flow rate of 1 mL min^{-1}. For analysis of the crude extract, substances were eluted with a linear gradient from 5–100% B in 30 min, washed with 100% (*v/v*) solvent B for 5 min and equilibrated with 5% (*v/v*) solvent B for 5 min. For analysis of the enzyme assays with **4**, substances were eluted with a linear gradient from 10–50% B in 20 min, washed with 100% (*v/v*) solvent B for 5 min and equilibrated with 10% (*v/v*) solvent B

for 5 min (method A). For analysis of enzyme assays with **5**, substances were eluted with a linear gradient from 30–100% B in 30 min, washed with 100% (*v/v*) solvent B for 5 min and equilibrated with 30% (*v/v*) solvent B for 5 min (method B). UV absorptions at 254 nm were illustrated. Semi-preparative HPLC was performed on the same equipment with an ODS column (C18, 9.4 × 250 mm, aters Pak, 5 μm) column and the flow rate of 2.5 mL min^{-1}.

The ethyl acetate extracts were analyzed on an Agilent HPLC 1260 series system equipped with a photo diode array detector and a Bruker microTOF QIII mass spectrometer by using a Multospher 120 RP-18 column (250x4mm, 5 μm, CS-Chromatograpie Service, Langerwehe, Germany). A linear gradient of 5−100% acetonitrile in water, both containing 0.1% formic acid, in 40 min and a flow rate at 0.25 mL min^{-1} were used. The column was then washed with 100% acetonitrile containing 0.1% formic acid for 5 min and equilibrated with 5% acetonitrile in water for 5 min. The parameters of the mass spectrometer were set as following: electrospray positive ion mode for ionization, capillary voltage with 4.5 kV, 1.

NMR analysis

^{1}H and ^{13}C NMR data were collected on a Bruker Avance-500 spectrometer using TMS as internal standard and chemical shifts were recorded as δ values. HR-ESI-MS detected on an Agilent Accurate-Mass-QTOF LC-MS 6520 instrument. Spectra were processed with MestReNova 6.1.0 (Metrelab Research, S5 Santiago de Compostella, Spain). NMR spectra and data of the cyclodipeptides are provided as **Figure S2–S3**.

Supplementary tables

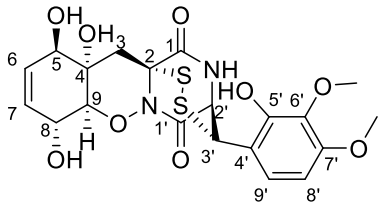
Table S1. Plasmids and strains used in this study

Plasmid/Strain	Description	Sources
Plasmids		
pET28a(+) SUMO	vector with T7 promoter, 6xHis tag, T7 terminator and SUMO tag	[6]
pUCH2-8	vector with hygromycin B (*hph*) resistance gene	[7]
pYHL79	*tdaR* ORF insert to pET28a(+)SUMO	This study
pYHL86	*gliT* ORF insert to pET28a(+)SUMO	This study
pYHL87	*aclT* ORF insert to pET28a(+)SUMO	This study
pYHL14	*tdaA* deletion cassette containing the *hph* selectable marker	This study
pYHL39	*tdaR* deletion cassette containing the *hph* selectable marker	This study
Strains		
E. coli DH5α	-	[4]
E. coli BL21(DE3)	-	[4]
Trichoderma hypoxylon CGMCC 3.17906	wild type (WT)	[1]
Aspergillus fumigatus Af293	WT	[8]
Aspergillus oryzae	WT	[9]
TYHL4	Δ*tdaA::hph* in *Trichoderma hypoxylon*	This study
TYHL23	Δ*tdaR::hph* in *Trichoderma hypoxylon*	This study

Table S2. Primers used in this study

Primer	**Sequences (5′-3′)**	
tdaR-QC-F	gaggctcacagagaacagattggtggtatgatatcgcgagctatgg	Amplification of *tdaR* ORF from the cDNA of *T. hypoxylon*
tdaR-QC-R	gcgccgaataaatacctaagcttgtcttcatataacccttttacattctg	
gliT-QC-F	gaggctcacagagaacagattggtggtatgtcgatcggcaaactactc	Amplification of *gliT* ORF from the cDNA of *A. fumigatus*
gliT-QC-R	gcgccgaataaatacctaagcttgtctttagctcctgatcgagacgaaac	
aclT-QC-F	acgatattattgaggctcacagagaacagattggtggtatggctgctccgctctttga	Amplification of *aclT* ORF from the cDNA of *A. oryzae*
aclT-QC-R	gacgcactttgcgccgaataaatacctaagcttgtctttttcgccttctcatcaaacgc	
tdaA-QC-5F-F	ctatagggcgaattggagctccaccgcggctcgtcagcagaatgtg	Up flanks' amplification for *tdaA* deletion
tdaA-QC-5F-R	gatccactagttctagagcggccgccaccgtccaccacttgcttgttcg	
tadA-QC-3F-F	gagccggaagcataaagtgtaaagcctggcctctatcactgtggcc	Down flanks' amplification for *tdaA* deletion
tdaA-QC-3F-R	gtgagttagctcactcattaggcacccgcactgcacgatatcagcc	
tdaA SCR F	gtcggatgcatcggcgtag	Upstream screening for Δ*tdaA* transformant verification
tdaA SCR R	caaccgtgctcactgcag	Downstream screening for Δ*tdaA* transformant verification
tdaA-RT F	gacacggtagtgttcggtg	Δ*tdaA* transformant target screening
tdaA-RT R	ggttgccatggtgtacag	
tdaR-QC-5F F	ctatagggcgaattggagctccaccgcgtactgctcggcagtctgg	Up flanks' amplification for *tdaR* deletion
tdaR-QC-5F R	gatccactagttctagagcggccgccaccggagttggcatacttcctcg	
tdaR-QC-3F F	gagccggaagcataaagtgtaaagcctgctgcggaactcaatcatggc	Down flanks' amplification for *tdaR* deletion
tdaR-QC-3F R	gtgagttagctcactcattaggcacccgagatggtcgaggaggcttc	
tdaR-SCR F	ccgcctgtagtgacatccag	Upstream screening for Δ*tdaR* transformant verification
tdaR-SCR R	gaggctaataactaccgtcgc	Downstream screening for Δ*tdaR* transformant verification
tdaR-RT F	gcctagctcgccaactacac	Δ*tdaR* transformant target screening
tdaR-RT R	ggaaccacagtcacctacag	

Table S3. ^{1}H and ^{13}C NMR data for pretrichodermamide A (**1**)



pretrichodermamide A (**1**) acquired in DMSO-d_6

NO.	δ_C, Type	δ_H (J in Hz)
1	167.0, C	-
2	70.8, C	-
3	30.7, CH_2	2.09 (d, 16.0) 1.96 (d, 16.0)
4	70.8, C	-
5	74.1, CH	4.17 (m)
6	129.8, CH	5.42 (br d, 10.4)
7	128.7, CH	5.48 (dt, 10.4, 2.4)
8	64.6, CH	4.23 (m)
9	85.5, CH	3.93 (d, 6.4)
1′	164.7, C	-
2′	58.9, CH	4.41 (dd, 4.2, 3.0)
3′	44.8, CH	4.49 (d, 2.8)
4′	116.5, C	-
5′	147.8, C	-
6′	135.8, C	-
7′	153.0, C	-
8′	103.3, CH	6.50 (d, 8.9)
9′	123.0, CH	7.44 (d, 8.8)
4-OH	-	5.09 (br s)
5-OH	-	5.26 (d, 5.1)
8-OH	-	5.22 (d, 6.7)
-NH	-	9.05 (d, 4.2)
7′-OCH_3	55.7, CH_3	3.67 (s)
8′-OCH_3	60.3, CH_3	3.78 (s)
9′-OH		9.43 (s)

Table S4. Gene prediction of *tda* cluster in *T. hypoxylon* by blast analysis

Trichoderma hypoxylon	***Trichoderma virens*** **GVW**	***Aspergillus oryzae*** **RIB40**	***Aspergillus fumigatus*** **Af293**	**Putative function**
tdaA (T_hypo_11188)	Glv21 (78/88)	BAE56606.1 (95/41)	XP_750855.1 (89/31)	Nonribosomal peptide synthase
tdaA0 (T_hypo_11189)	Glv20 (82/75)	—	—	Hypothetical protein
tdaB (T_hypo_11190)	Glv19 (78/83)	—	XP_750862.1 (99/40)	Cytochrome P450 oxygenase
tdaC (T_hypo_11191)	Glv18 (74/80)	BAE56607.1 (83/40)	—	Transporter
tdaD (T_hypo_11192)	Glv17 (95/98)	—	—	Dehydrogenase
tdaE (T_hypo_11193)	Glv16 (82/89)	BAE56600.1 (98/36)	—	FAD-dependent oxidoreductase
tdaF (T_hypo_11194)	Glv15 (82/90)	BAE56597.1 (98/45)	—	Aminotransferase
tdaG (T_hypo_11195)	Glv14 (81/85)	BAE56590.1 (83/40)	—	Cytochrome P450 oxygenase
tdaH (T_hypo_11196)	Glv13 (76/84)	—	—	Methyltransferase
tdaI (T_hypo_11197)	Glv12 (85/90)	BAE56591.1 (96/42)	—	Cytochrome P450 oxygenase
tdaJ (T_hypo_11198)	Glv11 (81/89)	BAE56605.1 (99/59)	XP_750854.1 (82/54)	Dipeptidase
tdaK (T_hypo_11199)	—	BAE56599.1 (99/48)	—	Methyltransferase
tdaL (T_hypo_11200)	Glv10 (70/79)	BAE56603.1 (88/62)	XP_750858.1 (84/38)	Glutathione-*S*-transferase
tdaM (T_hypo_11201)	Glv9 (86/92)	BAE56594.1 (98/39)	XP_750860.1 (97/28)	Transporter
tdaN (T_hypo_11202)	Glv8 (79/87)	BAE56592.1 (78/27)	XP_750852.1 (96/53)	Regulator
tdaO (T_hypo_11203)	Glv7 (76/84)	BAE56609.1 (100/48)	—	Methyltransferase
tdaP (T_hypo_11204)	Glv6 (83/88)	BAE56598.1 (93/42)	—	Cytochrome P450 oxygenase
tdaQ (T_hypo_11205)	Glv5 (80/88)	BAE56589.1 (90/35)	—	Cytochrome P450 oxygenase
tdaR (T_hypo_11206)	Glv4 (88/94)	BAE56601.1 (96/42)	XP_750863.1(98/38)	FAD-dependent oxidoreductase
tdaS (T_hypo_11207)	Glv3 (77/88)	BAE56604.1 (96/42)	XP_750856.1 (98/41)	Cytochrome P450 oxygenase
tdaT (T_hypo_11208)	Glv2 (84/92)	BAE56596.1 (98/54)	XP_750853.2 (94/37)	Aminotransferase
tdaU (T_hypo_11209)	Glv1 (88/94)	BAE56602.1 (100/56)	XP_750857.2 (96/46)	Methyltransferase
tdaV (T_hypo_11210)	—	—	XP_750859.1 (84/32)	Hypothetical protein

Supplementary figures

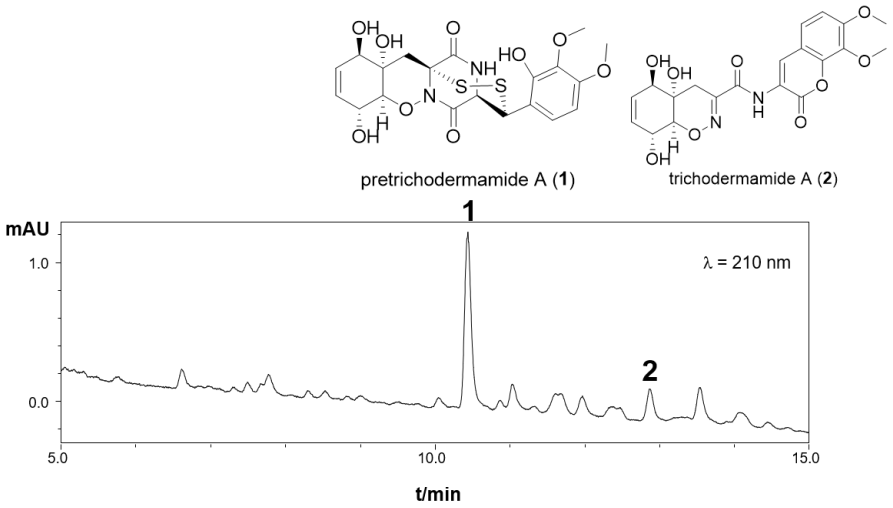


Figure S1. HPLC analysis of the crude extract of *T. hypoxylon* WT strain

UV absorptions at 210 nm are illustrated.

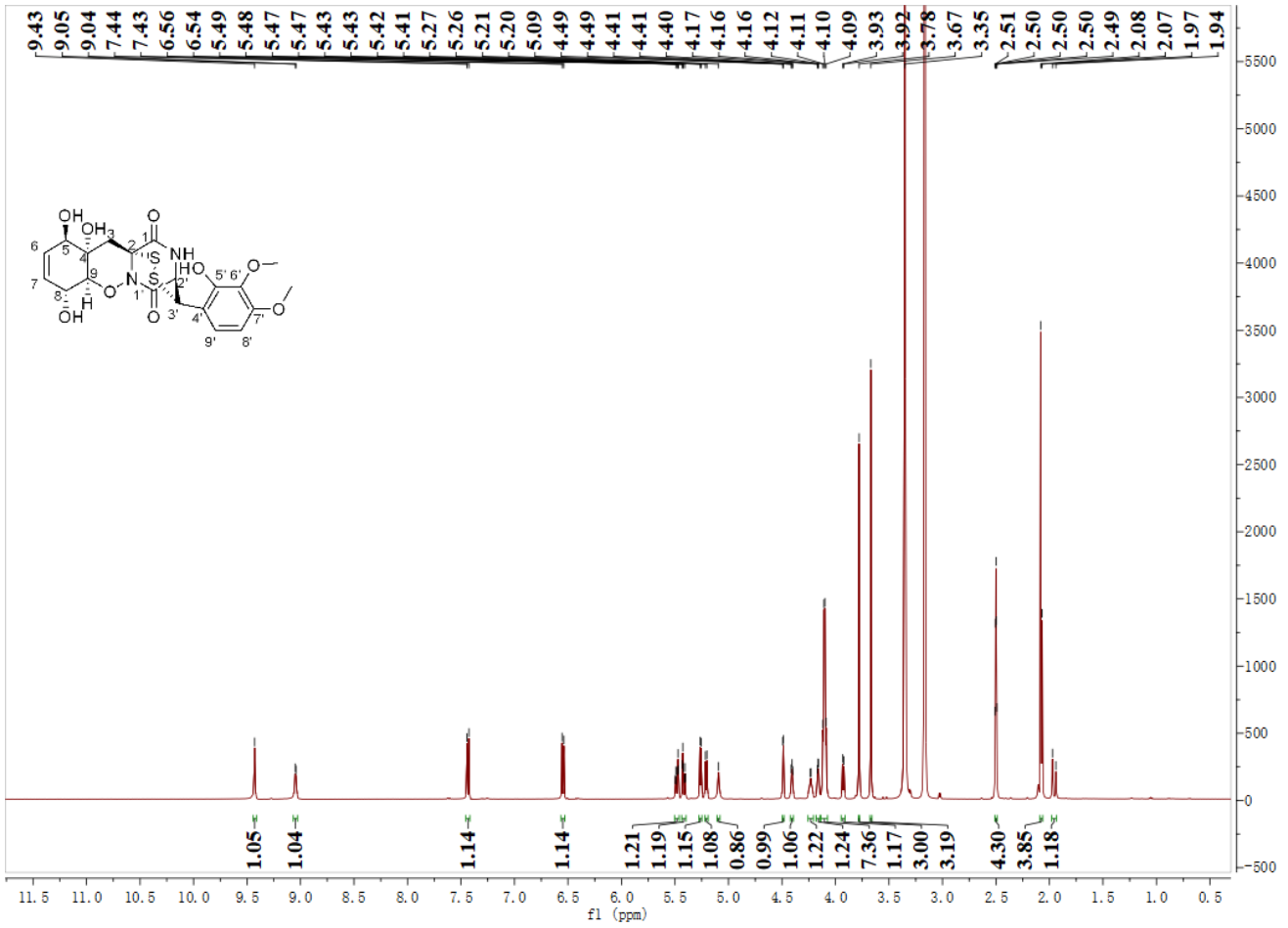


Figure S2. ^{1}H NMR spectrum of pretrichodermamide A (**1**) in DMSO-d_6 (500 MHz)

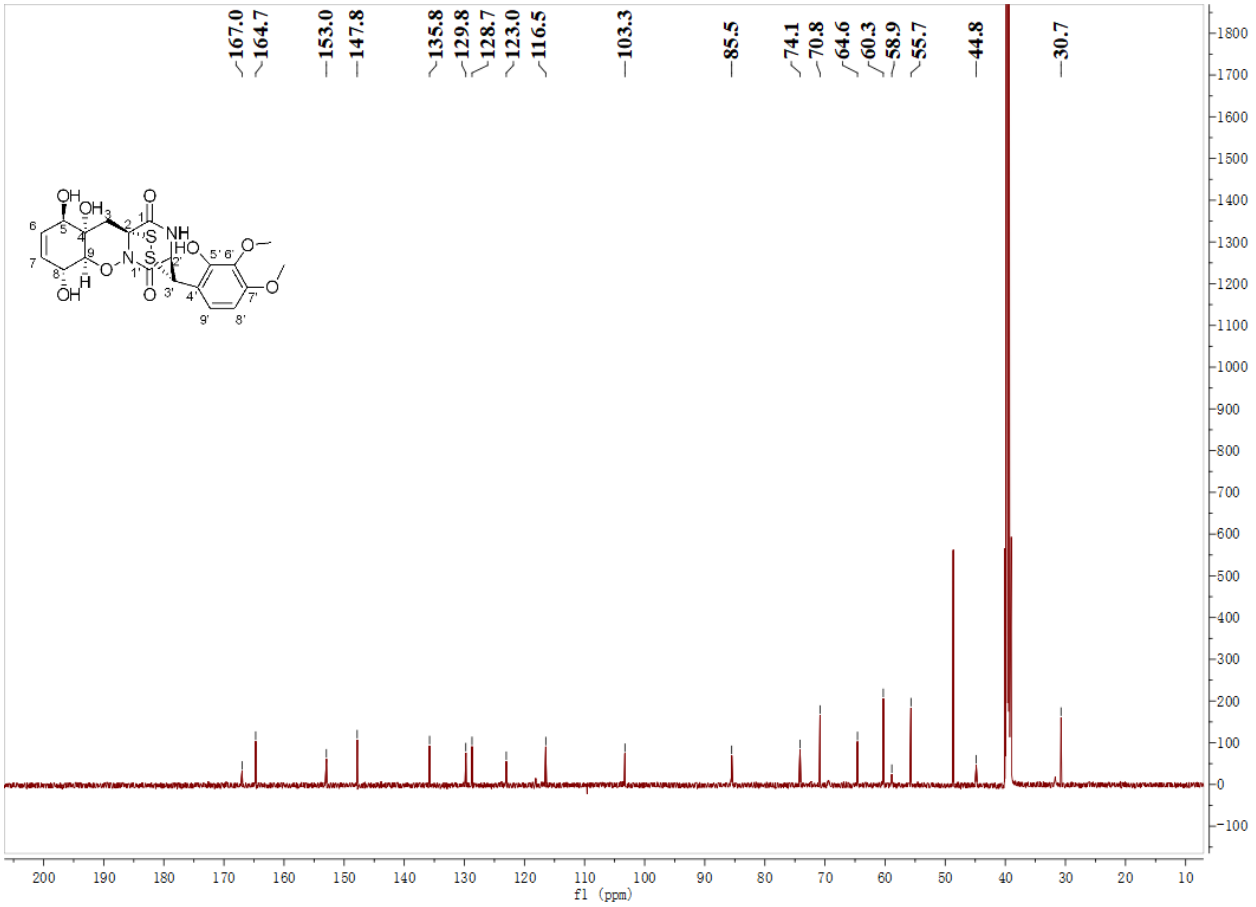


Figure S3. ^{13}C NMR spectrum of pretrichodermamide A (**1**) in DMSO-d_6 (125 MHz)

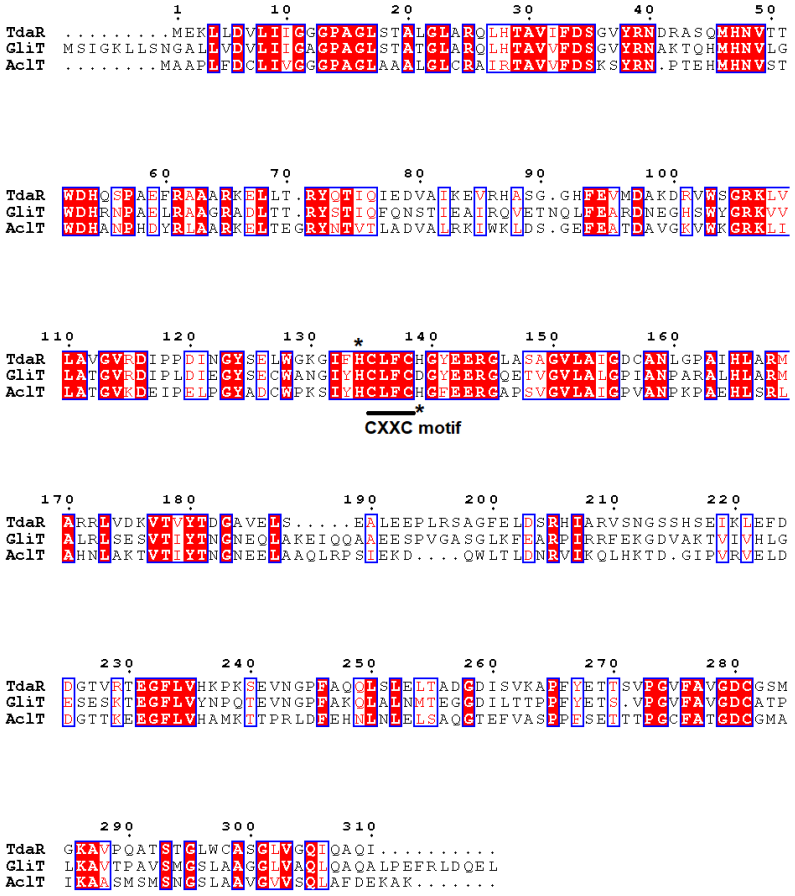


Figure S4. Sequence alignments of FAD-dependent oxidoreductases

TdaR (T_hypo_11206) from *T. hypoxylon* in this study shares conserved substrate binding site (His134, marked with *), CXXC motif box (Cys135-X-X-Cys138) and FAD activation site (His_{139}, marked with *) by comparing to GliT (XP_750863.1) from *A. fumigatus* and AclT (BAE56600.1) from *A. oryzae*. Protein sequence alignments were carried out by using the sequence alignment function of ClustalW and visualized with ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/)[2].

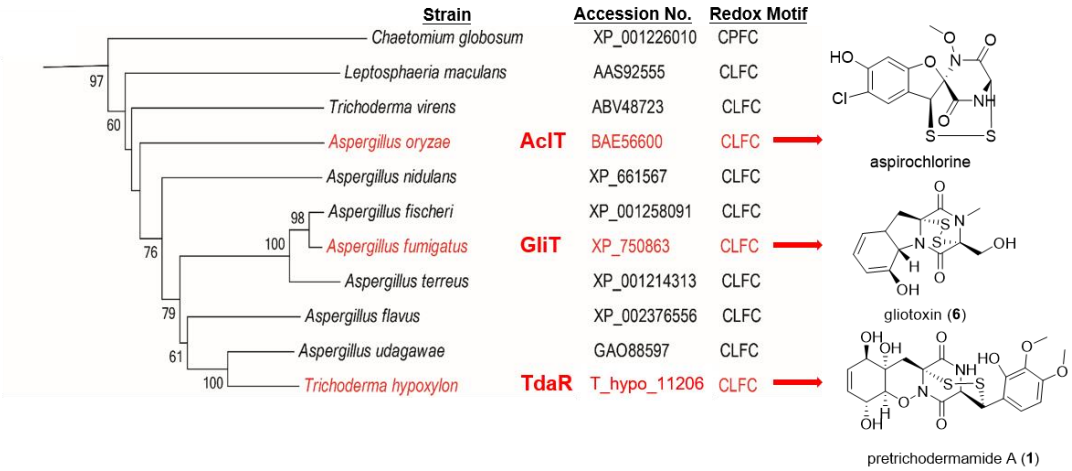


Figure S5. Phylogenetic analysis of TdaR homologs from fungal ETP clusters of clade IV in figure 3

The proteins from different fungi contain the same CLFC motif catalyzing α, α- or α, β-disulfide formation. TdaR, AclT and GliT were naturally responsible for the biosynthesis of pretrichodermamide A (**1**), aspirochlorine and gliotoxin (**6**), respectively.

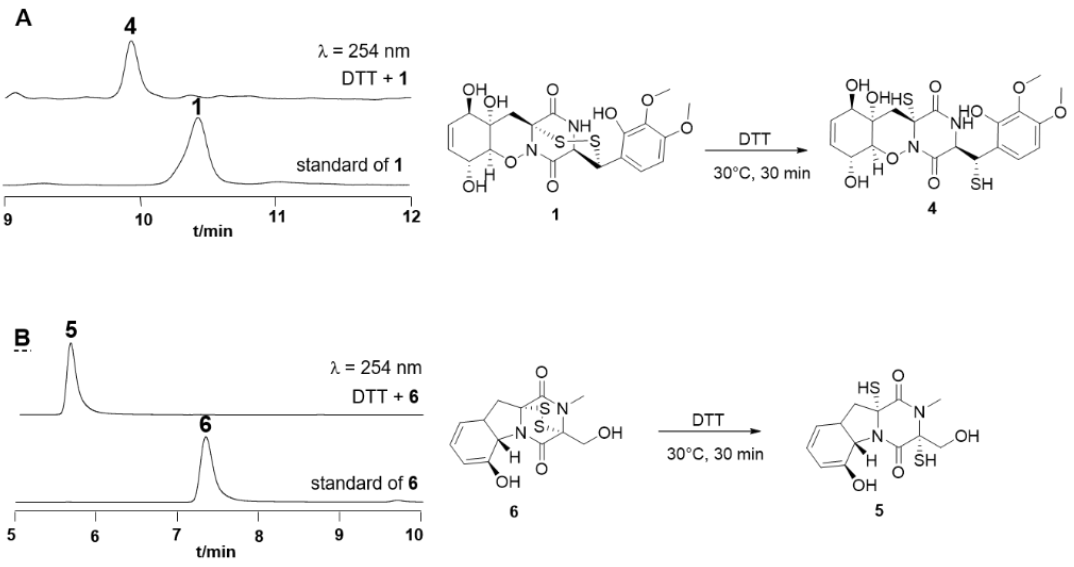


Figure S6. Reduction of pretrichodermamide A (**1**) (A) and gliotoxin (**6**) (B) by DTT

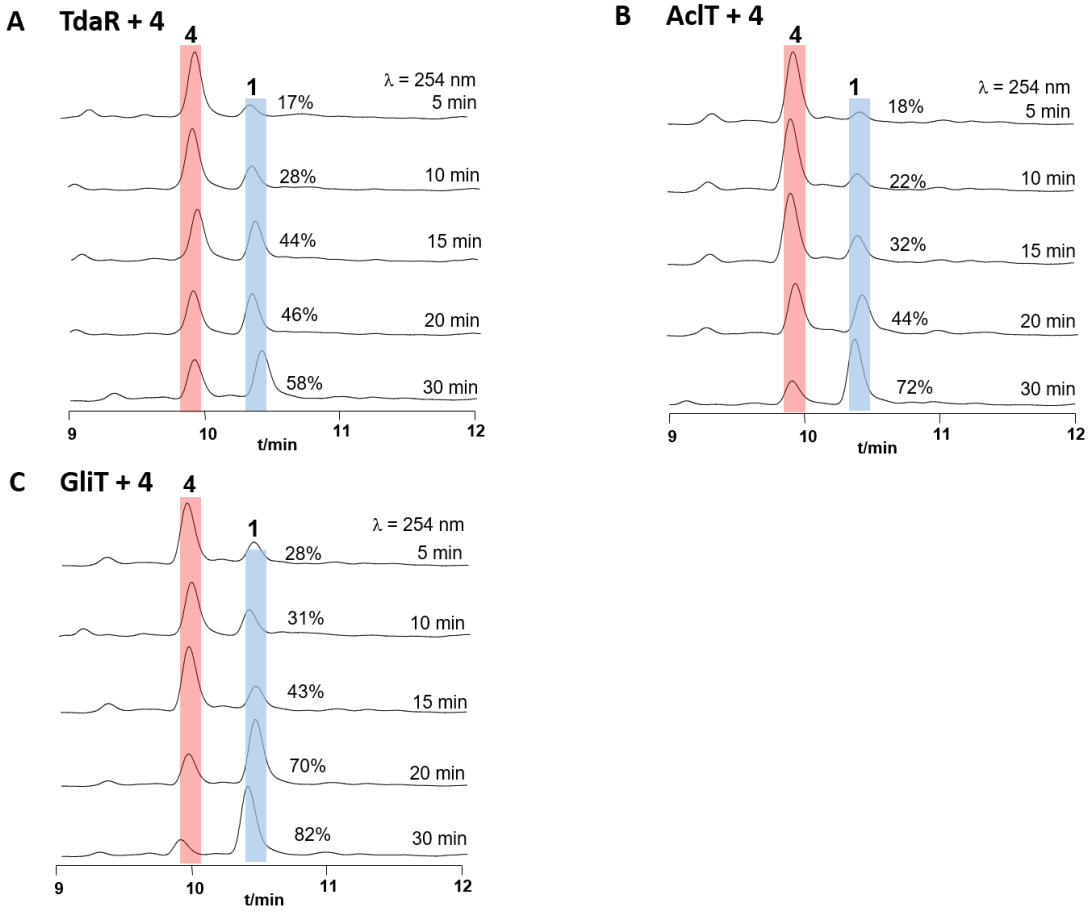


Figure S7. HPLC analysis of enzyme assays of TdaR, AclT and GliT with red-pretrichodermamide A (**4**) in a DTT reducing mixture

Enzyme reactions were terminated after 5, 10, 15, 20 and 30 min. UV absorptions at 254 nm are illustrated.

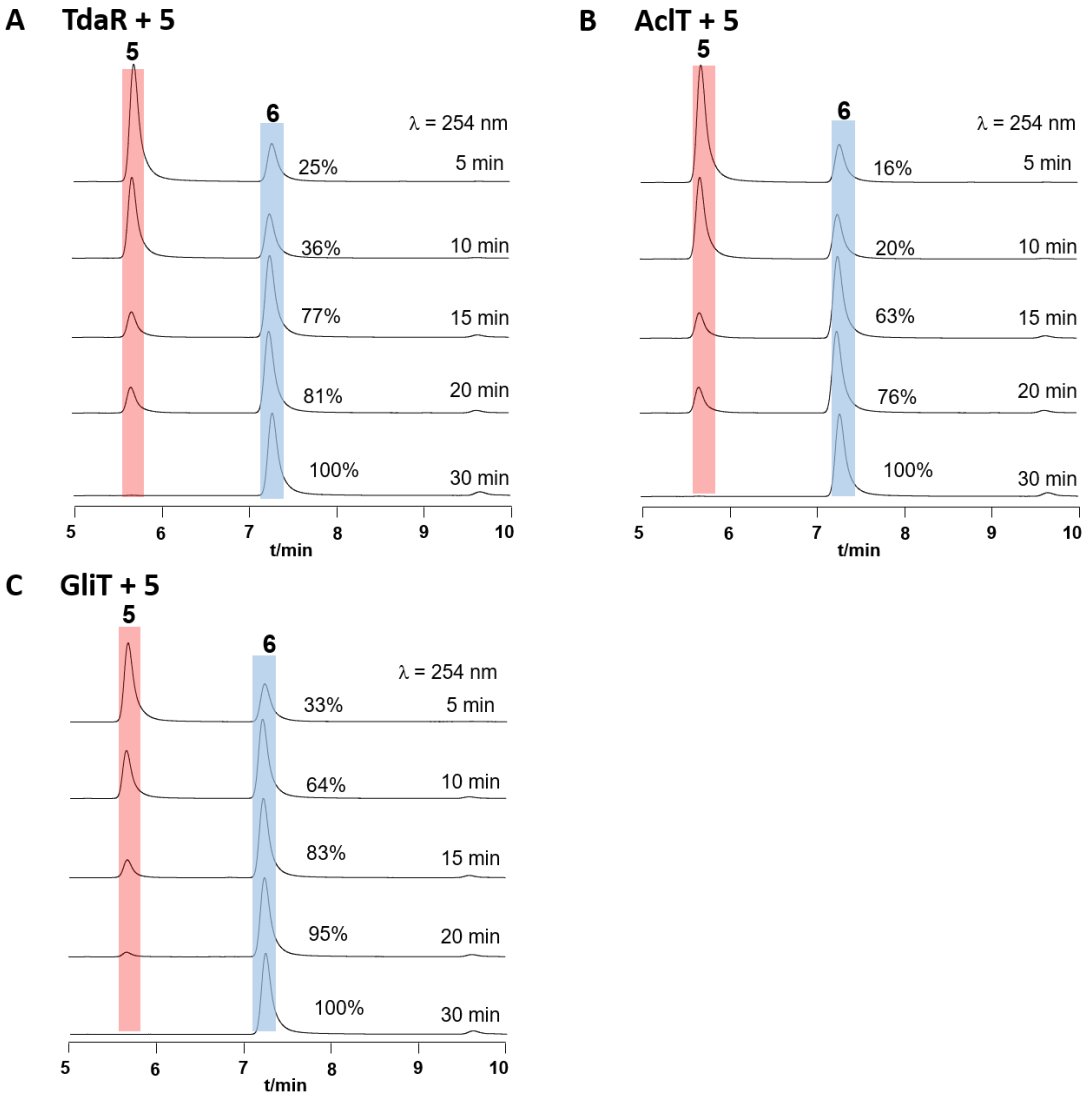


Figure S8. HPLC analysis of enzyme assays of TdaR, AclT and GliT with red-gliotoxin (**5**) in a DTT reducing mixture

Enzyme reactions were terminated after 5, 10, 15, 20 and 30 min. UV absorptions at 254 nm are illustrated.

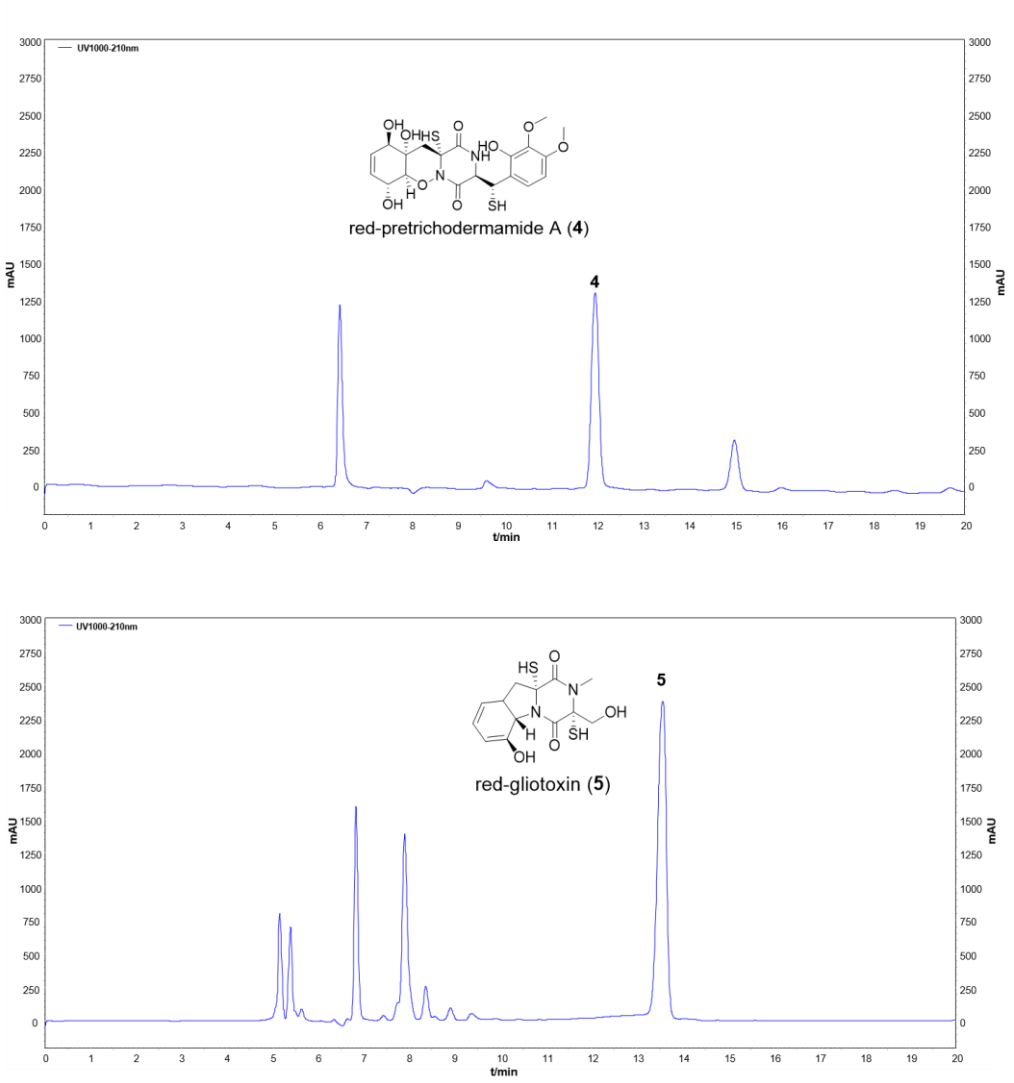


Figure S9. Preparation of red-pretrichodermamide A (**4**) and red-gliotoxin (**5**) on semi-preparative HPLC

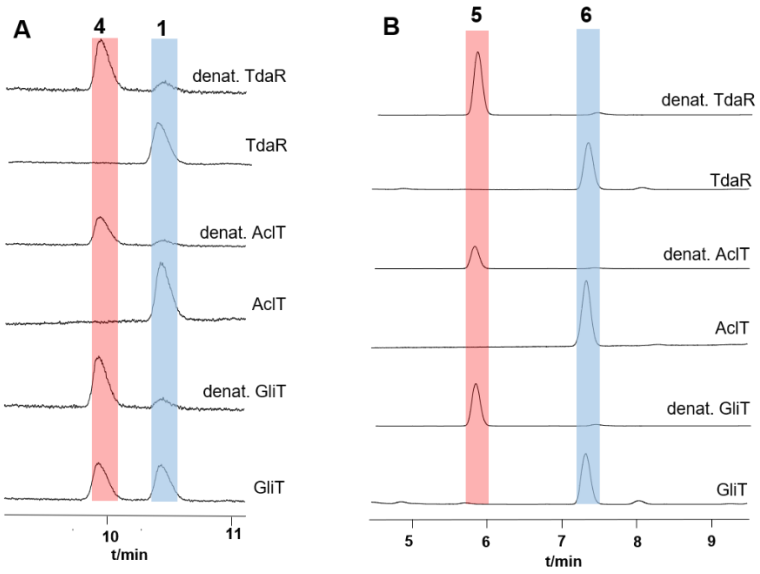


Figure S10. HPLC analysis of enzyme assays of TdaR, AclT and GliT with the pure substrates red-pretrichodermamide A (**4**) (A) and red-gliotoxin (**5**) (B)

Enzyme reactions were terminated after 30 min. UV absorptions at 254 nm are illustrated.

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