Electronic Supplementary Material

Macrophage-targeting oligopeptides from *Mortierella alpina*

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Experimental procedures

Isolation of genomic DNA

Young mycelia (4 d, MEP medium) from *M. alpina* ATCC32222 and *M. amoeboidea* CBS889.72 were ground under liquid nitrogen. DNA was isolated from 50 mg ground material using the DNeasy Plant mini kit (Qiagen) according to manufacturer's instructions except for the following adjustments: Tissue lysis at 65°C was extended up to 2 h, and 20 µg proteinase K (Merck) was added after 1 h of incubation. Eluted DNA from different 3 aliquots were pooled and DNA was extracted three times with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1, Carl Roth). DNA was precipitated with a double volume of ice-cold isopropanol at -20°C for 30 min. After centrifugation (14,000 g, 30 min), the pellet was washed 12 times with 70% ethanol, dried under airflow, and dissolved over night at 4°C in 10 mM TRIS buffer (pH 8.0). DNA quality was examined using a Scandrop photometer (Analytik Jena), for detection of impurities of protein or RNA, and a Tapestation 4150 (Agilent) for size distribution and integrity. Samples showing absorption values of $1.75 < A_{260/280} < 1.9$ and $A_{260/230} > 2.0$ and a mean fragment size above 20 kbp were chosen for genome sequence-library preparation and ITS sequencing.

Strain identification

Strains were identified by sequencing the internal transcribed spacer (ITS) regions. The two ITS regions of ribosomal DNA were amplified from genomic DNA using the oligonucleotides oITS1/oITS4 (Table S14) and Phusion DNA polymerase (Thermo) according to the manufacturer's instructions. PCR products were sequenced.

Genome sequencing and annotation

Libraries were generated from 400 ng DNA using the SQK-RAD004 kit (Oxford Nanopore Technologies, ONT) following the manufacturer's instructions except for the use of nuclease free water instead of "loading beads". Sequencing was carried out on a MinION using a FLO-MIN106D (ONT) (R9 chemistry). After 24 h, a nuclease-digest was performed utilizing the EXP-WSH003 (ONT) flow cell wash kit and sequencing was continued with a new generated library. Raw reads were basecalled with GUPPY v.3.2.6 GPU version. Genomes were assembled using CANU[1, 2] v.1.9. assuming a genome size of 40 Mbp based on genomes related *Mortierella* isolates accessed through JGI Mycosom[3] and an available *M. alpina* genome.[4]

To improve assemblies, signal level reads were indexed to the draft genomes by Nanopolish[5] and after sorting and mapping using minimap[6] and SAMtools[7], an improved consensus sequence was calculated using Nanopolish[8]. Genes were annotated using the AUGUSTUS Web server.[9] The *Rhizopus oryzae* genome was chosen as reference and genes were identified on both strands. Genome completeness was check by BUSCO[10] using the option "auto-lineage_euk" resulting in the automated selection of the "mucorales_odb10" dataset.

Identification of genes and expression analysis

The assembled genomes of *M. alpina* and *M. amoeboidea* were screened for NRPS genes using the antiSMASH 5.0 website.[11] NRPS domain borders were manually adjusted using Pfam[12] and clustal W2

alignment tools[13] using fungal and bacterial NRPS as templates. Intron/exon borders were identified with the AUGUSTUS annotation tool.[9]

RNA from mycelium was isolated using SV Total RNA Isolation System (Promega) and gDNA was digested with Baseline-Zero DNAse (Biozym). cDNA synthesis was carried out with RevertAid Reverse Transcriptase (Thermo Fisher), RiboLock RNase inhibitor (Thermo Fisher) and anchored oligo(dT)$_{18}$ primers. Oligonucleotides for candidate NRPS genes (*nps2*, *nps3* (*malA*) and *nps5*) as well as the two housekeeping genes *actB* (encoding β-actin) and *gpdA* (encoding glycerinaldehyde-3-phosphate dehydrogenase) were designed, and PCR efficiency was checked to be at least 95% (Table S14). For analysis of the malpinin biosynthetic gene cluster, additional primer pairs for five genes adjacent to *malA* were included (*orf27, orf29, orf32, orf34, orf35*). qRT-PCR (quantitative real-time PCR) was performed on the qPCR cycler qTower^3 (Analytik Jena) using the qPCR Mix EvaGreen (BioSELL) following the manufacturer's instructions. The following qPCR protocol was run: 15 min initial denaturation 95°C, 40 cycles of amplification (95°C, 15 s; 60°C, 20 s; 72°C, 20 s) and a final monitoring of a melting curve from 60 to 95°C in 1°C increments.

Cloning procedure

A first set of oligonucleotides was used to amplify the coding sequences (CDS) of the modules 2-7 (C-A-T tri-domains) of *malA* from *M. alpina* cDNA (Table S14) by PCR. Using these fragments as templates, the respective restriction sites at the 5' and 3' flank were introduced by a second PCR run with an additional set of oligonucleotides (Tables S14). Phusion high fidelity DNA polymerase (NEB) was used according to manufacturer's protocol and the following gradient was applied: initial denaturation 98°C, 2 min; 40 cycles of amplification (98°C, 20 s; 60°C, 20 s; 72°C, 180 s); final extension 72°C, 5 min. After blunt end ligation into pJET1.2 cloning vector (Thermo Fisher), fragments were excised from purified plasmids using the restriction enzymes listed in Table S14 and were ligated with an accordingly digested pET28a (+) vector using the T4-DNA-Ligase (NEB).

The CDS of module 1 of *malA* was amplified using oligonucleotides oMG270 and oMG285 (Table S11) and its pET28a vector backbone was amplified using oligonucleotides oMG283 and oMG284. Both fragments were ligated using the In-Fusion® HD Cloning Plus kit (TaKaRa).

The ligation mixtures were used to transform *Escherichia coli* XL1 blue to propagate the plasmids (Table S15) and *E. coli* expression strains (Table S3).

Heterologous protein production and purification

E. coli expression strains were cultivated in 500 mL LB medium containing 100 µg mL^{-1} kanamycin (CarlRoth) at 37°C until an OD_{600} of 0.4-0.6 was reached. Subsequently, the incubation temperature was set to 16°C and gene expression was induced by addition of 0.1% L-rhamnose (Carl Roth) for *E. coli* KRX and 1 mM isopropyl-β-D-1-thiogalactopyranoside for *E. coli* strains BL21 and SoluBL21. After cultivation overnight, cells were harvested by centrifugation (3,200 × g, 40 min, 4°C) and the cell pellet was resuspended in lysis buffer (20 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride, pH 8.0). Lysis was accomplished by sonification with a Sonoplus ultrasonic sonifier (Bandelin) (5 cycles with 20 s impulse interval and 5 s impulse duration; 0.8 kJ total impulse energy). The N- and C-terminal His_6-tagged fusion proteins were purified by gravity flow metal ion affinity chromatography (Protino Ni-NTA agarose, Macherey-Nagel). The column was washed with lysis buffer and increasing concentrations of imidazole (40- and 80-mM). Tagged enzymes were eluted in lysis buffer with 250 mM imidazole. Proteins were desalted and concentrated by Amicon Ultra-15 centrifugal filter units 100 kDa

(Merck) and rebuffered in protein storage buffer (50 mM TRIS, 200 mM sodium chloride, 10% glycerol). Protein concentrations were determined according to Bradford with the Protein Assay Kit (BioRad).[14] Proteins were stored at -80°C.

Determination of the *N*-Acetylation

Synthesis of aminoacyl-*N*-acetylcysteamine (SNAC) thioesters

Aminoacyl SNACs were synthesised as described before.[15] In brief, 2 mmol of *N*-protected amino acids (*N*-Boc-L-leucine, *N*-Boc-D-leucine, *N*-acetyl-L-leucine, and *N*-acetyl-D-leucine), 2 mmol *N,N'*-dicyclohexylcarbodiimide (DCC), 2 mmol hydroxybenzotriazole (HOBt) and 2 mmol *N*-acetylcysteamine were solved in 30 mL THF and stirred at room temperature for 1h. After addition of 1 mmol K_2CO_3, the mixture was stirred for another 3h. The reaction was filtered and evaporated to dryness. The residue was dissolved in ethyl acetate and extracted twice with 10% aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4, evaporated and dissolved in dichloromethane (CH_2Cl_2). Purification was performed by flash chromatography (Büchi Pure C-810) using isocratic conditions (4% MeOH in CH_2Cl_2) and a 12g Silica column. Separation was monitored by UHPLC-MS (method B, Table S15) and fractions containing *N*-acetyl-leucyl-SNAC or *N*-Boc-leucyl-SNAC thioesters were pooled. Boc-protected products were deprotected by acidic treatment in 6 mL 50% TFA/CH_2Cl_2 for 1 h. TFA was removed by repeated addition and evaporation of CH_2Cl_2 *in vacuo*. Finally, the residues were dissolved in approx. 500 - 1000 µL of CH_2Cl_2 and precipitated with 9 mL diethyl ether. Precipitates was filtered, washed with diethyl ether and finally dried. All SNAC thioesters were dissolved in 1% aqueous TFA and stored at -80°C up to 3 days.

N*-Acetylation of leucyl-SNAC thioesters *in vitro

N-Acetylation of leucyl-SNAC thioesters was performed using a modified protocol according to Zhong et al.[16] In a total volume of 100 µL, heterologously produced MalA-M1 (10 µM) or *M. alpina* crude protein extract (400 µg mL^{-1}, determined by Bradford assay) were mixed with 500 µM acetyl-CoA and 2.5 mM L- or D-leucyl-SNAC in reaction buffer (50 mM Tris-HCl, 25 mM NaCl, 10 mM $MgCl_2$, pH 7.5). As controls, Ac-CoA was omitted from the reactions. After incubation for 30 min at 37°C, reactions were stopped by addition of 100 µL MeOH and centrifuged at 20.000 × g for 10 min. To prevent degradation of SNAC-thioesters, samples were measured instantly by UHPLC-MS using method B (Table S15). Separately synthesised *N*-acetyl-L-leucyl-SNAC and *N*-acetyl-D-leucyl-SNAC served as product standards.

To generated crude protein extract, mycelium of *M. alpina* was harvested from liquid cultures under malpinin-inducing conditions (LB medium, 160 rpm, 25 °C, 4 days) and ground under liquid nitrogen. The fungal powder was resuspended in reaction buffer and insoluble cell debris were removed by centrifugation (20.000 × g for 10 min). The protein concentration of the supernatant was determined by Bradford assay (ThermoFisher).

Microscopical imaging

Leukocyte concentrates were prepared from peripheral blood obtained from healthy human adult donors (Institute of Transfusion Medicine, University Hospital Jena, Germany). The approval for the protocol was given by the ethical committee of the University Hospital Jena and all methods were

performed in accordance with the relevant guidelines and regulations. The leukocyte concentrates were mixed with dextran (dextran from *Leuconostoc* spp. MW ~40,000) for sedimentation of erythrocytes; the supernatant was centrifuged on lymphocyte separation medium (Histopaque®-1077). Contaminating erythrocytes in the pelleted neutrophils were removed by hypotonic lysis using water. Neutrophils were then washed twice in ice-cold phosphate-buffered saline (PBS) and finally resuspended in PBS. The peripheral blood mononuclear cell (PBMC) fraction on top of the lymphocyte separation medium was washed with ice-cold PBS and seeded in cell culture flasks (Greiner Bio-one, Nuertingen, Germany) for 1.5 h (37 °C, 5% CO_2) in PBS with Ca^{2+}/Mg^{2+} to isolate monocytes by adherence. For the differentiation of monocytes towards macrophages, we followed published procedures.[17] Thus, adherent monocytes were treated with 20 ng mL^{-1} granulocyte macrophage-colony stimulating factor (GM-CSF) (Peprotech, Hamburg, Germany) for 6 days in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}) to get monocyte-derived macrophages. The purity of these macrophages was routinely checked by flow cytometry (LSRFortessa™ cell analyzer, BD Biosciences, Heidelberg, Germany) as reported[18] using the following antibodies: FITC anti-human CD14 (clone M5E2, BD Biosciences), APC-H7 anti-human CD80 (clone L307.4, BD Biosciences), PE-Cy7 anti-human CD54 (clone HA58, Biolegend, Koblenz, Germany), PE anti-human CD163 (clone GHI/61, BD Biosciences), and APC anti-human CD206 (clone 19.2, BD Biosciences).

HEK293 cells (ATCC, Manassas, US) were cultured in monolayers (37 °C, 5% CO_2) in DMEM containing FCS (10%), penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}).

Microscopy and treatment with FAM and 27

Macrophages (1×10^6 cells), neutrophils (2×10^6 cells) and HEK cells (0.5×10^6 cells) were seeded onto polylysine-coated glass coverslips in 12-well plates and cultured at 37°C for 1 h for neutrophils and 24 h for macrophages and HEK cells. FAM, FAM-coupled malpinin (**27**) or vehicle (DMSO) were added and macrophages were incubated for 3 h and neutrophils and HEK cells for 1 h at 37 °C. Cells were then fixed using 4% paraformaldehyde solution. Cells were stained with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA, United States). Samples were analyzed by a Zeiss Axiovert 200M microscope with a Plan Neofluar × 40/1.30 Oil (DIC III) objective (Carl Zeiss, Jena, Germany) and the 38HE-green fluorescent reflector to measure FAM intensity. An AxioCam MR camera (Carl Zeiss) was used for image acquisition.

Phylogenetic analysis

Phylogenetic analysis of fungal and bacterial A domains was performed as described by Wurlitzer *et al*.[19] A set of 123 A domains were extracted from NRPSs, NRPS-like proteins (including the α-2-aminoadipate reductases, aryl acid reductases, tyrosine reductases, and serine reductases), and PKS/NRPS hybrids from *M. alpina* (MalA, MpcA and MpbA), (endo)bacteria, and higher fungi. The A domain of the tryptophanyl-tRNA synthetase Wrs1 from *Saccharomyces cerevisiae* served as the outgroup. The percentual bootstrap support was calculated for 1000 replicates.

Structural modelling of the starter condensation domain MalA-C_s

The N-terminal domain of MalA has been structurally modelled with AlphaFold v2.1.0[20] using a publicly available script on Google Colab (AlphaFold.ipynb). The model has been superimposed with a crystal structure of the C_s domain from CDA biosynthesis (PDB 4JN3, grey cartoon) in Pymol v2.2.2 using the "super" command.

Table S1. Comparison of *M. alpina* and *M. amoeboidea* genomes. [#] According to BUSCO analysis.[10]

	M. alpina ATCC32222	*M. alpina* ATCC32222	*M. amoeboidea* CBS889.72
Genbank accession	GCA_000240685.2	JALIRG010000000	JALIGY010000000
BioSample	SAMN02981246	SAMN27177060	SAMN27177162
BioProject	PRJNA41211	PRJNA817090	PRJNA817100
sequencing method	pyrosequencing and Sanger sequencing	nanopore sequencing	nanopore sequencing
total sequence length	38,042,092	39,295,276	38,155,153
number of contigs	1,099	43	24
completeness[#]		90.4%	94.8%
coverage	31.8x	39.7x	144.0x
contig N50	171,448	2,196,674	2,511,580
contig L50	70	7	6
contig N90	44,192	811,690	1,667,963
contig L90	235	16	14
reference	[4]	This study.	This study.

Table S2. Identified NRPS genes in *M. alpina* and *M. amoeboidea*. The protein domain architecture is presented. A, adenylation domain; C canonical condensation domain; Cs, starter condensation domain; E/C, dual epimerisation/condensation domain; T, thiolation domain; TE, thioesterase domain; TD, terminal reductase domain. Highly homologous NRPS genes in both species (>70% identity) are highlighted in green.

	***M. alpina* ATCC32222**	modules	encoding contig	gene
Nps1	Cs-A-T-C-A-T-E/C-A-T-E/C-A-T-C-A-T-C-A-T-E/C-A-T-C-A-T-E/C-A-T-TE	9	tig00000075	
Nps2	A-T-E/C-A-T-C-A-T-E/C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-TE	8	tig00000075	
Nps3	Cs-A-T-E/C-A-T-E/C-A-T-E/C-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	7	tig00000075	*malA*
Nps4	Cs-A-T-C-A-T-C-A-T-E/C-A-T-E/C-A-T-E/C-A-T-C-A-T-TE	7	tig00002287	
Nps5	Cs-A-T-E/C-A-T-C-A-E/C-A-T-E/C-A-T-C-A-T-TE	6	tig00080470	
Nps6	Cs-A-T-C-A-T-C-A-T-C-A-T-C-A-T-E/C-A-T-TE	6	tig00080470	
Nps7	Cs-A-T-E/C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-TE	6	tig00000137	
Nps8	Cs-A-T-E/C-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	5	tig00002279	*mpcA*
Nps9	Cs-A-T-E/C-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	5	tig00002279	*mpbA*
Nps10	Cs-A-T-E/C-A-T-C-A-T-C-A-T-TD	4	tig00000022	
Nps11	Cs-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	4	tig00002287	
Nps12	Cs-A-T-C-A-T-C-A-T-C-A-T-TE	4	tig00080466	
Nps13	Cs-A-T-C-A-T-TE	2	tig00002287	
Nps14	A-T-C-T-C-A-TD	?	tig00000022	
Nps15	Cs-A-T-TE	1	tig00002287	
Nps16	A-T-TD	1	tig00000005	*lys2*

	***M. amoeboidea* CBS 889.72**	modules	encoding contig	homologs in *M.alpina* genome		Protein identity
Nps1	Cs-A-T-C-A-T-C-A-T-E/C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-E/C-A-T-TE	9	tig00000044	*nps1*		43.59%
Nps2	A-T-E/C-A-T-C-A-T-E/C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-TE	8	tig00000028	*nps2*		72.81%
Nps3	Cs-A-T-E/C-A-T-E/C-A-T-E/C-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	7	tig00000028	*nps3*	*malA*	90.20%
Nps4	Cs-A-T-C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-TE	7	tig00000038	*nps4*		63%
Nps5	Cs-A-T-E/C-A-T-C-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	6	tig00000033	*nps5*		89.16%
Nps6	Cs-A-T-E/C-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	5	tig00000040	*nps8*	*mpcA*	90.04%
Nps7	Cs-A-T-E/C-A-T-C-A-T-E/C-A-T-E/C-A-T-TE	5	tig00000040	*nps9*	*mpbA*	84.88%
Nps8	A-T-E/C-A-T-E/C-A-T-E/C-A-T-E/C-A-T-TE	5	tig00000030	-		
Nps9	A-T-E/C-A-T-C-A-T-C-A-T-TD	4	tig00000002	*nps10*		84.44%
Nps10	A-T-C-A-T-C-A-T-C-A-T-TE	4	tig00000111	*nps12*		78.76%
Nps11	T-C-A-T-C-A-T-C-A-T-C-A-T-TD	4	tig00000028	-		
Nps12	Cs-A-T-E/C-A-T-C-A-T-TE	3	tig00000044	-		
Nps13	A-T-C-A-T-TE	2	tig00000028	*nps13*		42.97%
Nps14	C-A-T-TD	1	tig00000028	-		
Nps15	A-T-TD	1	tig00000002	-		
Nps16	A-T-?	1	tig00000040	-		
Nps17	A-T-TD	1	tig00013576	*nps16*	*lys2*	94.09%

Table S3. Heterologously produced proteins after Ni^{2+}-NTA-affinity chromatography. Yields refer to a 500 ml bacterial culture.

MalA-module	plasmid	*E. coli* expression strain	His_6-tag	protein MW	yield [mg]
M1	pMG027	*E. coli* KRX	N+C-terminal	110.9 kDa	0.070
M2	pJMW31	*E. coli* SoluBL21	N+C-terminal	120.1 kDa	0.226
M3	pJMW33	*E. coli* SoluBL21	N+C-terminal	119.6 kDa	0.180
M4	pJMW10	*E. coli* KRX	N+C-terminal	123.8 kDa	0.420
M5	pJMW24	*E. coli* KRX	N+C-terminal	123.1 kDa	0.062
M6	pJMW27	*E. coli* BL21	N+C-terminal	125.5 kDa	0.180
M7	pJMW26	*E. coli* SoluBL21	N+C-terminal	120.2 kDa	0.044

Table S4. Quantification of aminoacyl hydroxamates in adenylation domain assays. Values are expressed in µM.

	M1		M2		M3		M4		M5		M6		M7	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
D-PheHA	0.076	0.003	0	0	0.541	0.020	0.258	0.019	0	0	0.038	0.001	0	0
L-PheHA	0	0	0	0	0.157	0.003	18.797	0.246	0.040	0.001	0.781	0.029	0.054	0.000
L-HisHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-MetHA	1.508	0.004	0	0	19.638	0.128	1.358	0.072	0	0	0	0	0	0
L-GluHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-LysHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-AspHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-IlleHA	0.028	0.000	0	0	0	0	0	0	0	0	0	0	0	0
L-LeuHA	100.000	0.000	0	0	100.000	0.000	0.178	0.005	0	0	0.071	0.002	0	0
L-CysHA	0.127	0.019	0	0	2.083	0.194	0	0	0	0	0	0	0	0
L-ThrHA	0	0	0.073	0.011	0	0	0	0	68.825	0.594	0	0	0	0
D-ValHA	0	0	0	0	9.866	0.269	0	0	0.252	0.004	0	0	0	0
L-ValHA	0.091	0.004	0	0	1.700	0.211	0	0	0	0	0	0	0	0
L-ProHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GlyHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-TrpHA	0	0	0	0	0	0	1.453	0.101	0	0	792.672	5.572	0	0
L-TyrHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-ArgHA	0	0	15.201	2.153	0	0	0	0	0	0	0	0	0	0
L-AlaHA	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S5. Substrate specificity code for MalA adenylation domains and related domains.
The proposed activated substrates are listed in column 3. The residues are mapped based on positions according to *Aneurinibacillus migulanus* (former *Brevibacillus brevis)* GrsA-A numbering [21]. NRPS codes were extracted from various sources.[22-24] Amino acid residues in the NRPS code are highlighted according to their physicochemical properties: acidic (red), small/hydrophobic (grey), aromatic/hydrophobic (ocher), hydrophilic (green), and basic (blue).

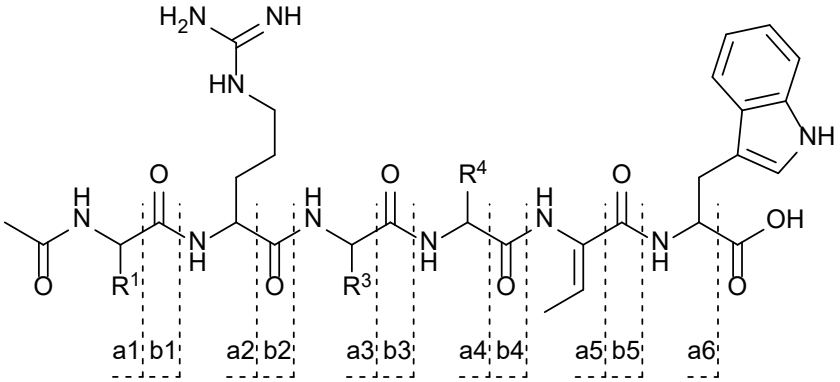
Protein domain	Organism*	substrate	Residue position according to GrsA Phe numbering									
			235	236	239	278	299	301	322	330	331	527
MalA_A1	*M. alpina*	Leu/Val/Met	D	F	W	N	F	G	L	I	Y	K
MalA_A1	*M. amoeboidea*	Leu/Val	D	F	W	N	F	G	L	I	Y	K
MalA_A3	*M. alpina*	Leu/Val/Met	D	F	W	A	A	G	L	I	A	K
MalA_A3	*M. amoeboidea*	Leu/Val	D	F	W	A	S	G	I	I	A	K
MpbA_A1	*M. alpina*	Val	D	A	F	W	L	G	G	T	F	K
MpcA_A1	*M. alpina*	Leu/Ile/Val	D	A	F	F	I	G	A	M	L	K
MpbA_A2	*M. alpina*	Leu	D	A	F	F	I	G	A	M	V	K
MpbA_A4	*M. alpina*	Leu	D	A	I	F	L	G	A	T	I	K
MpcA_A5	*M. alpina*	Leu	D	A	M	F	I	G	G	T	I	K
Plu3263_A2	*P. luminescens*	Leu	D	A	W	C	I	G	A	V	C	K
SrfAA_A2	*B. subtilis*	Leu	D	A	F	M	M	G	M	V	F	K
GrsB_A1	*A. migulanus*	Val	D	A	F	W	I	G	G	T	F	K
EasA_A3	*A. nidulans*	Leu	D	I	H	F	V	G	A	I	A	K
BSLS_A2	*B. bassiana*	Leu	D	G	Y	I	I	G	G	V	F	K
CssA_A9	*T. inflatum*	Val	D	A	W	M	F	A	A	V	L	K
MalA_A2	*M. alpina*	Arg	D	F	W	T	T	G	D	I	S	K
MalA_A2	*M. amoeboidea*	Arg	D	F	W	T	T	G	D	I	S	K
MpcA_A3	*M. alpina*	Arg	D	A	A	S	V	G	A	T	D	K
SyrE_A5	*P. syringae*	Arg	D	V	A	D	V	C	A	I	D	K
McyC_A1	*M. aeruginosa*	Arg	D	V	W	T	I	G	A	V	D	K
McyB_A1	*M. aeruginosa*	Arg	D	V	W	T	I	G	A	V	E	K
PpzA-1_A2	*E. festucae*	Arg	D	V	S	D	T	G	A	P	T	K
MalA_A4	*M. alpina*	Phe	D	P	W	I	L	G	E	I	A	K
MalA_A4	*M. amoeboidea*	Phe	D	P	W	I	L	G	E	I	A	K
MpcA_A2	*M. alpina*	Tyr/Phe	D	P	F	T	M	G	A	V	V	K
MpbA_A3	*M. alpina*	Tyr/Phe/Trp	D	P	F	V	M	G	G	T	V	K
Plu3263_A3	*P. luminescens*	Phe	D	A	W	C	I	A	A	V	C	K
BacC_A2	*B. subtilis*	Phe	D	A	F	T	V	A	A	V	C	K
GrsA_A1	*A. migulanus*	Phe	D	A	W	T	I	A	A	I	C	K
TycA_A1	*A. migulanus*	Phe	D	A	W	T	I	A	A	I	C	K
CepA_A1	*A. orientalis*	Tyr	D	A	S	T	V	A	A	V	C	K
TycC_A1	*A. migulanus*	Tyr	D	A	L	T	T	G	E	V	V	K
GliP_A1	*A. fumigatus*	Phe	D	G	S	I	L	G	A	C	A	K
BEAS_A2	*B. bassiana*	Phe	D	G	Y	I	M	A	A	V	M	K
MalA_A5	*M. alpina*	Thr/Dhb	D	F	W	N	V	G	M	V	H	K
MalA_A5	*M. amoeboidea*	Thr/Dhb	D	F	W	N	V	G	M	V	H	K
HgdA_A1	*B. gladioli*	Dhb	D	F	W	N	V	G	M	V	H	K
HgdA_A2	*B. gladioli*	Dhb	D	F	W	N	V	G	M	V	H	K
AcmB_A1	*S. chrysomallus*	Thr	D	F	W	N	V	G	M	V	H	K
SnbC_A1	*S. pristinaespiralis*	Thr	D	F	W	N	V	G	M	V	H	K
SyrB_A1	*P. syringae*	Thr	D	F	W	S	V	G	M	V	H	K
PvdD_A1	*P. aeruginosa*	Thr	D	F	W	N	I	G	M	V	H	K
BgdA_A1	*B. gladioli*	Dhb	D	F	W	N	I	G	M	V	H	K
AgiA_A1	*A. flavus*	Thr	D	A	G	A	M	G	T	V	D	K
MalA_A6	*M. alpina*	Trp	D	A	L	A	I	G	E	V	A	K
MalA_A6	*M. amoeboidea*	Trp	D	A	L	A	I	G	E	V	A	K
DaptA_A1	*S. roseosporus*	Trp	D	V	S	S	I	G	A	V	E	K
TqaA_A2	*P. aethiopicum*	Trp	D	G	M	H	V	V	G	V	S	K
IvoA_A1	*A. nidulans*	Trp	D	V	D	L	Q	G	V	V	A	K
MalA_A7	*M. alpina*	inactive	D	P	L	I	L	G	E	I	A	K
MalA_A7	*M. amoeboidea*	inactive	D	P	L	I	L	G	E	I	A	K

*Organisms are highlighted according to their phylogenetic origin: basal fungi (blue), higher fungi (brown), and bacteria (green).

Table S6. Properties of metabolites determined in this study. DOI, degrees of unsaturation; Dhb, dehydrobutyrine; pCys, *S*-propargyl-L-cysteine; BrPhe, 4-bromo-L-phenylalanine; FAM, 5-carboxyflourescein; N_3Phe, 4-azido-L-phenylalanine; t_R, retention time according to UHPLC-MS analysis (method B and method C, Table S16). Met-containing metabolites (**6-17**) other malpinins (**1-5** and **18-27**) were quantified by method B and method C, respectively. Asterisks (*) indicates metabolite structure elucidation by ESI-MS/MS, absolute configuration was not determined. For respective MS/MS spectra see Figures S12, S15-S23, S38-S39, S41, S43, S44, S52 and S53. n.d., not determined.

cmpd.	name	$[M+H]^+$	formula	DOI	composition	t_R (min) meth. B	t_R (min) meth. C
1	Malpinin A	859.4827	$C_{44}H_{62}N_{10}O_8$	19	Ac-D-Leu-D-Arg-D-Leu-L-Phe-Dhb-D-Trp	2.65	9.91
2	Malpinin B	845.4665	$C_{43}H_{60}N_{10}O_8$	19	Ac-D-Leu-D-Arg-D-Val-L-Phe-Dhb-D-Trp	2.50	9.26
3	Malpinin C	845.4674	$C_{43}H_{60}N_{10}O_8$	19	Ac-D-Val-D-Arg-D-Leu-L-Phe-Dhb-D-Trp	2.50	9.26
4	Malpinin D	831.4515	$C_{42}H_{58}N_{10}O_8$	19	Ac-D-Val-D-Arg-D-Val-L-Phe-Dhb-D-Trp	2.37	8.69
5	Malpinin E*	825.4971	$C_{41}H_{65}N_{10}O_8$	15	Ac-Leu-Arg-Leu-Leu-Dhb-Trp	2.54	n.d.
6	Malpinin F	877.4368	$C_{43}H_{60}N_{10}O_8S$	19	Ac-D-Leu-D-Arg-D-Met-L-Phe-Dhb-D-Trp	n.d.	9.38
7	Malpinin G	877.4374	$C_{43}H_{60}N_{10}O_8S$	19	Ac-D-Met-D-Arg-D-Leu-L-Phe-Dhb-D-Trp	n.d.	9.38
8	Malpinin H*	895.3948	$C_{42}H_{58}N_{10}O_8S_2$	19	Ac-Met-Arg-Met-Phe-Dhb-Trp	n.d.	8.74
9	Malpinin I*	843.4547	$C_{40}H_{62}N_{10}O_8S$	15	Ac-Leu-Arg-Leu-Met-Dhb-Trp	n.d.	8.96
10	Malpinin J*	829.4379	$C_{39}H_{60}N_{10}O_8S$	15	Ac-Leu-Arg-Val-Met-Dhb-Trp	n.d.	8.33
11	Malpinin K*	829.4379	$C_{39}H_{60}N_{10}O_8S$	15	Ac-Val-Arg-Leu-Met-Dhb-Trp	n.d.	8.33
12	Malpinin L*	815.4222	$C_{38}H_{58}N_{10}O_8S$	15	Ac-Val-Arg-Val-Met-Dhb-Trp	n.d.	7.75
13	Malpinin M*	861.4098	$C_{39}H_{60}N_{10}O_8S_2$	15	Ac-Leu-Arg-Met-Met-Dhb-Trp	n.d.	8.43
14	Malpinin N*	861.4098	$C_{39}H_{60}N_{10}O_8S_2$	15	Ac-Met-Arg-Leu-Met-Dhb-Trp	n.d.	8.43
15	Malpinin O*	847.3946	$C_{38}H_{58}N_{10}O_8S_2$	15	Ac-Met-Arg-Val-Met-Dhb-Trp	n.d.	7.84
16	Malpinin P*	847.3946	$C_{38}H_{58}N_{10}O_8S_2$	15	Ac-Val-Arg-Met-Met-Dhb-Trp	n.d.	7.84
17	Malpinin Q*	879.3653	$C_{38}H_{58}N_{10}O_8S_3$	15	Ac-Met-Arg-Met-Met-Dhb-Trp	n.d.	7.87
18	Malpinin R*	898.4911	$C_{46}H_{63}N_{11}O_8$	21	Ac-Leu-Arg-Leu-Trp-Dhb-Trp	2.59	n.d.
19	Malpinin S*	884.4770	$C_{45}H_{61}N_{11}O_8$	21	Ac-Leu-Arg-Val-Trp-Dhb-Trp	2.45	n.d.
20	Malpinin T*	884.4770	$C_{45}H_{61}N_{11}O_8$	21	Ac-Val-Arg-Leu-Trp-Dhb-Trp	2.45	n.d.
21	Malpinin U*	887.42244	$C_{44}H_{58}N_{10}O_8S$	21	Ac-pCys-Arg-Leu-Phe-Dhb-Trp	2.54	n.d.
22	Malpinin V*	887.42244	$C_{44}H_{58}N_{10}O_8S$	21	Ac-Leu-Arg-pCys-Phe-Dhb-Trp	2.54	n.d.
23	Malpinin W	937.39196	$C_{44}H_{61}N_{10}O_8Br$	19	Ac-Leu-Arg-Leu-BrPhe-Dhb-Trp	2.79	n.d.
24	Malpinin X*	923.37666	$C_{43}H_{59}N_{10}O_8Br$	19	Ac-Leu-Arg-Val-BrPhe-Dhb-Trp	2.67	n.d.
25	Malpinin Y*	923.37666	$C_{43}H_{59}N_{10}O_8Br$	19	Ac-Val-Arg-Leu-BrPhe-Dhb-Trp	2.67	n.d.
26	-	900.48357	$C_{44}H_{61}N_{13}O_8$	21	Ac-Val-Arg-Leu-N_3Phe-Dhb-Trp	2.66	n.d.
27	Malpinin-coupled 5-FAM	1313.5734	$C_{68}H_{76}N_{14}O_{14}$	38	Ac-Leu-Arg-Leu-(Phe-triazole-5-FAM)-Dhb-Trp	2.51	n.d.
Deacetyl-**1**	Deacetyl-Malpinin A*	817.4716	$C_{42}H_{60}N_{10}O_7$	18	Leu-Arg-Leu-Phe-Dhb-Trp	2.25	n.d.

Table S7. HR-ESI-MS/MS data of compounds 1 and 6-27. The N-terminal charged fragment ions are indicated as a and b. The subscript n denotes the number of amino acid residues. Compound fragmentation for **1** served as reference.[25] * Fragments of low abundance.



	R^1	R^3	R^4	a_1	b_1	a_2	b_2	a_3	b_3	a_4	b_4	a_5 +2H	b_5	a_6
Malpinin A (**1**)	Leu	Leu	Phe	128.11	-	284.21	-	397.29	-	544.36	-	629.41	-	813.47
Malpinin F (**6**)	Leu	Met	Phe	128.11	156.10	284.21	312.20	415.25	443.24	562.32	590.31	647.37	673.35	831.43
Malpinin G (**7**)	Met	Leu	Phe	146.06	174.06	302.16	330.16	415.25	443.24	562.32	590.31	647.37	673.35	831.44
Malpinin H (**8**)	Met	Met	Phe	146.06	174.06	302.16	330.16	433.21	461.2	580.27	608.27	665.33	691.3	849.39
Malpinin I (**9**)	Leu	Leu	Met	128.11	156.1	284.21	312.2	397.29	425.28	528.33	556.33	613.39	639.36	797.45
Malpinin J (**10**)	Leu	Val	Met	128.11	156.1	284.21	312.2	383.28	411.27	514.32	542.31	599.37	625.35	783.43
Malpinin K (**11**)	Val	Leu	Met	114.09	142.09	270.18	298.19	383.28	411.27	514.32	542.31	599.37	625.35	783.43
Malpinin L (**12**)	Val	Val	Met	114.09	142.09	270.19	298.19	369.29	397.26	500.3	528.3	585.35	611.33	769.42
Malpinin M (**13**)	Leu	Met	Met	128.11	156.1	284.21	312.2	415.25	443.24	546.29	574.28	631.34	657.32	815.41
Malpinin N (**14**)	Met	Leu	Met	146.06	174.06	302.16	330.16	415.25	443.24	546.29	574.28	631.34	657.32	815.41
Malpinin O (**15**)	Met	Val	Met	146.06	174.06	302.16	330.16	401.23	429.23	532.27	560.27	617.33	643.31	801.39
Malpinin P (**16**)	Val	Met	Met	114.09	142.09	270.19	298.19	401.23	429.23	532.27	560.27	617.33	643.31	801.39
Malpinin Q (**17**)	Met	Met	Met	146.06	174.06	302.16	330.16	433.21	461.2	564.25	592.24	649.3	675.28	833.36
Malpinin R (**18**)	Leu	Leu	Trp	128.11	156.1	284.21	312.2	397.29	425.29	583.37	611.37	668.42	694.4	852.49
Malpinin S (**19**)	Leu	Val	Trp	128.11	156.1	284.21	312.2	383.28	411.27	569.36	597.35	654.41	680.39	838.47
Malpinin T (**20**)	Val	Leu	Trp	114.09	142.09	270.18	298.19	383.28	411.27	569.36	597.35	654.41	680.39	838.47
Malpinin U (**21**)	pCys	Leu	Phe	156.05 *	184.04	312.15*	340.14	425.23	453.23	572.3	600.3	657.35	683.33	841.42
Malpinin V (**22**)	Leu	pCys	Phe	128.11	156.1	284.21	312.2	425.23	453.23	572.3	600.3	657.35	683.33	841.42
Malpinin W (**23**)	Leu	Leu	BrPhe	128.11	156.1	284.21	312.2	397.29	425.29	622.27	650.27	707.32	733.3	891.39
Malpinin X (**24**)	Leu	Val	BrPhe	128.11	156.1	284.21	312.2	383.28	411.27	608.26	636.25	693.29	719.29	877.37
Malpinin Y (**25**)	Val	Leu	BrPhe	114.09	142.09	270.18	298.19	383.28	411.27	608.26	636.25	693.29	719.29	877.37
Malpinin azide (**26**)	Leu	Leu	N_3Phe	128.11	156.1	284.21	312.2	397.29	-	545.36 (-N_3)	571.34 (-N_3)	-	654.37 (-N_3)	827.47 (-N_2)
Malpinin-coupled 5-FAM (**27**)	Leu	Leu	Phe-5-FAM	128.11	156.1	284.21	312.2	397.29	425.29	998.44	1026.45	1081.48 (a5)	1109.48	1267.56
Deacetyl-**1**	Leu	Leu	Phe	86.10	114.09*	242.20*	270.19	355.28	383.28	502.35	530.35	-	613.38	-

Table S8. Malpinins in cultures supplemented with single amino acids. *M. alpina* was cultivated in Aspergillus Minimal Medium with supplementation of 10 mM proteinogenic amino acids **(A)** Leu, Val, Ile, Phe and Cys, **(B)** Met and Trp or **(C)** non-proteinogenic amino acids pCys and BrPhe. Yields are expressed as signal intensity per fungal dry biomass [AUC /g mycelium]. BrPhe, 4-bromo-L-phenylalanine, pCys, S-propargyl-L-cysteine; SD, standard deviation (n=3). See also: Figures 4 and S40.

A

		ctrl		Leu		Val		Ile	
cmpd.	**name**	**yield**	**SD**	**yield**	**SD**	**yield**	**SD**	**yield**	**SD**
1	malpinin A	2.89E+08	7.08E+07	2.33E+07	1.31E+07	2.01E+08	2.85E+07	1.33E+08	1.88E+07
2-3	malpinin B-C	1.24E+08	3.21E+07	7.14E+05	9.52E+04	1.84E+08	1.59E+07	9.20E+07	1.82E+07
4	malpinin D	1.07E+07	3.43E+06	0.00E+00	0.00E+00	4.01E+07	1.67E+06	4.74E+06	1.32E+06
5	malpinin E	2.17E+06	7.14E+05	8.54E+06	4.57E+06	4.22E+07	8.67E+06	1.45E+06	4.14E+05
6-7	malpinin F-G	-	-	-	-	-	-	-	-
8	malpinin H	-	-	-	-	-	-	-	-
9	malpinin I	-	-	-	-	6.66E+05	1.30E+05	-	-
10-11	malpinin J-K	-	-	-	-	-	-	-	-
12	malpinin L	-	-	-	-	-	-	-	-
13-14	malpinin M-N	-	-	-	-	-	-	-	-
15-16	malpinin O-P	-	-	-	-	-	-	-	-
17	malpinin Q	-	-	-	-	-	-	-	-
18	malpinin R	-	-	-	-	-	-	-	-
19-20	malpinin S-T	-	-	-	-	-	-	-	-
deacetyl-1	deacetyl-malpinin A	1.71E+06	9.88E+05	7.22E+05	8.91E+05	1.78E+06	2.74E+05	1.65E+06	4.73E+05

		Phe		Cys		LB-Medium	
cmpd.	**name**	**yield**	**SD**	**yield**	**SD**	**yield**	**SD**
1	malpinin A	7.63E+06	1.27E+06	3.58E+06	4.47E+05	5.19E+06	4.99E+04
2-3	malpinin B-C	5.18E+06	1.17E+06	2.09E+06	3.01E+05	1.68E+06	6.88E+03
4	malpinin D	6.79E+05	1.49E+05	2.09E+05	3.35E+04	3.45E+05	2.38E+04
5	malpinin E	-	-	-	-	1.64E+05	8.21E+03
6-7	malpinin F-G	-	-	-	-	1.11E+05	5.65E+03
8	malpinin H	-	-	-	-	-	-
9	malpinin I	-	-	-	-	2.07E+04	6.16E+03
10-11	malpinin J-K	-	-	-	-	-	-
12	malpinin L	-	-	-	-	-	-
13-14	malpinin M-N	-	-	-	-	-	-
15-16	malpinin O-P	-	-	-	-	-	-
17	malpinin Q	-	-	-	-	-	-
18	malpinin R	-	-	-	-	-	-
19-20	malpinin S-T	-	-	-	-	-	-
deacetyl-1	deacetyl-malpinin A	-	-	-	-	8.37E+04	5.01E+03

B

		Met		Trp	
cmpd.	**name**	**yield**	**SD**	**yield**	**SD**
1	malpinin A	2.05E+08	2.39E+07	2.19E+08	1.64E+08
2-3	malpinin B-C	1.18E+08	2.31E+07	1.03E+08	8.10E+07
4	malpinin D	2.04E+07	6.59E+06	2.11E+07	1.28E+07
5	malpinin E	-	-	1.17E+06	3.95E+05
6-7	malpinin F-G	2.29E+08	1.31E+08	3.17E+06	2.14E+06
8	malpinin H	6.56E+07	4.47E+07	-	-
9	malpinin I	1.38E+08	6.06E+07	-	-
10-11	malpinin J-K	5.05E+07	2.25E+07	-	-
12	malpinin L	4.34E+06	1.94E+06	-	-
13-14	malpinin M-N	1.34E+08	7.04E+07	-	-
15-16	malpinin O-P	3.02E+07	1.61E+07	-	-
17	malpinin Q	2.67E+07	1.58E+07	-	-
18	malpinin R	-	-	1.62E+07	1.25E+07
19-20	malpinin S-T	-	-	8.96E+06	6.08E+06

C

		ctrl		pCys		BrPhe	
cmpd.	**name**	**yield**	**SD**	**yield**	**SD**	**yield**	**SD**
1	malpinin A	1.70E+09	8.83E+08	1.99E+08	1.29E+08	714235078	44809821.2
2-3	malpinin B-C	7.98E+08	4.26E+08	1.16E+08	7.87E+07	357457616	32725988.5
4	malpinin D	1.06E+08	5.40E+07	1.59E+07	1.12E+07	46902840.8	7042333.95
5	malpinin E	1.01E+07	3.83E+06	-	-	-	-
21-22	malpinin U-V	-	-	4.75E+07	3.33E+07	-	-
23	malpinin W	-	-	-	-	335822906	47885510.7
24-25	malpinin X-Y	-	-	-	-	151904122	26360990.4

Table S9. NMR data of 6 and 7 in DMSO-d_6. For detailed atom numbering see Figure S34. * Signals overlap.

malpinin F (6)			**malpinin G (7)**		
pos.	δ_C [ppm]	δ_H [ppm], M (*J* [Hz])	pos.	δ_C [ppm]	δ_H [ppm], M (*J* [Hz])
tryptophan (Trp)			tryptophan (Trp)		
1	173.3		1	173.3	
2	53.4	4.47, q (7.9, 13.5)	2	53.4	4.47, q (7.9, 13.1)
2 NH	-	7.85, d (7.5)	2 NH	-	7.84, d (7.4)
3	26.8	a: 3.12, m *	3	26.8	a: 3.21, d (4.6)
		b: 3.20, d (4.8)			b: 3.13, m *
4	109.8	-	4	109.8	-
5	123.7	7.18, d (2.1)	5	123.7	7.18, m (8.0)
5 NH	-	10.84, d (1.8)	5 NH	-	10.85, s
6	127.1	-	6	127.1	-
7	118.0	7.53, d (7.8)	7	118.0	7.53, d (8.0)
8	118.3	6.98, t (7.4)	8	118.3	6.98, t (7.4)
9	120.9	7.05, t (7.5)	9	120.9	7.06, t (7.4)
10	111.4	7.33, d (8.0)	10	111.4	7.33, d (8.0)
11	136.1	-	11	136.1	-
dehydrobutyrine (Dhb)			dehydrobutyrine (Dhb)		
12	164.3	-	12	164.2	-
13	130.2	-	13	130.1	-
13 NH	-	9.18, s	13 NH	-	9.12, s
14	128.0	6.32, q (7.1, 14.1)	14	128.1	6.33, q (6.8, 13.8)
15	13.1	1.53, d (7.0)	15	13.1	1.53, d (6.9)
phenylalanine (Phe)			phenylalanine (Phe)		
16	170.0	-	16	170.2	
17	54.1	4.65, m	17	54.3	4.59, m
17 NH		8.34, d (8.0)	17 NH	-	8.36, d (7.7)
18	37.4	2.77, dd (11.0, 13.4)	18	37.3	2.78, dd (10.8, 13.4)
19	137.6	-	19	137.7	-
20	129.2	7.26, m *	20	129.3	7.27, d (7.3)
21	128.0	7.24, m *	21	128.0	7.24, t (7.1)
22	126.3	7.17, m *	22	126.2	7.18, m *
23	128.0	7.24, m *	23	128.0	7.24, t (7.1)
24	129.2	7.26, m *	24	129.3	7.27, d (7.3)
methionine (Met)			leucine (Leu)		
25	170.8	-	25	172.1	-
26	51.8	4.30, q (7.7, 12.6)	26	51.0	4.26, m *
26 NH	-	7.79, d (7.6)	26 NH	-	7.80, d (7.6)
27	32.0	a: 1.68, m *	27	41.0	1.16, q (7.3, 13.2)
		b: 1.52, m *	28	23.8	1.23, m *
28	29.0	2.09, t (15.8)	29	21.7	0.71, d (6.4)
29	14.4	1.92, s	30	22.8	0.74, d (6.4)
arginine (Arg)			arginine (Arg)		
30	171.1	-	31	171.0	
31	52.0	4.20, m *	32	52.02	4.23, m *
31 NH	-	8.07, d (7.7)	32 NH	-	8.06, d (7.5)
32	28.4	a: 1.65, m *	33	28.6	a: 1.61
		b: 1.48, m *			b: 1.47
33	25.0	a: 1.39, m *	34	25.0	1.38, m *
		b: 1.47, m *			1.45 m *
34	40.0	3.05, m *	35	40.4	3.05, m
34 NH	-	7.54, m *	35 NH	-	7.56, m *
35	156.6	-	36	156.7	-
leucine (Leu)			methionine (Met)		
36	172.5	-	37	171.5	-
37	51.1	4.22, m *	38	51.98	4.29, m *
37 NH	-	8.02, d (7.6)	38 NH	-	8.10, d (7.4)
38	40.4	1.39, m *	39	31.8	1.75, m (5.3)
39	24.1	1.58, m*	40	29.5	2.42, m (6.6)
40	23.0	0.86, d (6.9)	41	14.5	2.00, s
41	21.5	0.81, d (6.5)			
acetic acid (Ac)			acetic acid (Ac)		
42	169.4	-	42	169.5	-
43	22.4	1.82, s	43	22.4	1.83, s

Table S10. Determination of the absolute configuration of 6, 7 and 23 by advanced Marfey´s method. Retention times [min] of respective FDLA products are highlighted in red (L-stereoisomer) and blue (D-isomer). An authentic standard for (*Z*)-Dhb is not available due to instability (reactivity of α,β-unsaturated carbonyl moiety).

amino acid	$M_{AA\text{-}FDLA}$ [g/mol]	L-standard	D-standard	malpinin F (6)	malpinin G (7)	malpinin W (23)
leucine	426	7.70	9.08	9.10	9.10	9.10
arginine	469	5.44	5.30	5.32	5.35	5.30
methionine	444	7.05	8.14	8.16	8.16	-
phenylalanine	460	7.86	8.87	7.87	7.86	-
threonine	414	5.62	6.66	-	-	-
tryptophan	499	7.81	8.62	8.64	8.65	8.64
4-Br-phenylalanine	538	8.66	9.65	-	-	8.65

Table S11. NMR data of 23. For atom numbering see Figure S51. * signals overlapping

malpinin W (23)

pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])
tryptophan		
1	173.3	-
2	-	7.85, d (7.43)
2 NH	53.4	4.47, m*
3	26.8	B: 3.22, m*
		A: 3.13, m*
4	109.9	-
5	123.8	7.19, d (1.6)
5 NH	-	10.84, m
6	127.1	-
7	118.1	7.52, d (7.80)
8	118.3	6.98, m*
9	120.9	7.04, m*
10	111.4	7.33, d (8.07)
11	136.1	-
dehydrobutyrine		
12	164.3	-
13	130.2	-
13 NH		9.13
14	127.9	6.32, q (6.9)
15	13.1	1.55, d (7.0)
4-bromo-phenylalanine		
16	169.9	-
17	53.9	4.62, m (11.5)
17 NH	-	8.37, d (8.3)
18	36.7	A: 2.72, dd (11.1, 13.6)
		B: 3.10, m*
19	137.2	-
20, 24	131.6	7.27, d (8.3)
21, 23	130.9	7.43, d (8.1)
22	119.5	-

pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])
leucine		
25	172.0	-
26	51.06	4.21, m*
26 NH	-	7.72, d (7.5)
27	41.1	1.11, m*
28	23.8	1.15, m*
29	22.8	0.73, d (6.1)
30	21.7	0.71, d (6.3)
arginine		
31	170.9	
32	51.9	4.22, m*
32 NH		8.04, d (8.0)
33	28.5	A: 1.48, m*
		B: 1.63, m*
34	25.0	A: 1.45, m *
		B: 1.39, m *
35	40.4	3.05, m*
35 NH	-	7.52, m*
36	156.7	
leucine		
37	172.5	-
38	51.14	4.24, m*
38 NH		8.02, d (7.6)
39	40.6	1.38, m*
40	24.1	1.58, m *
41	23.0	0.85, d (6.4)
42	21.6	0.81, d (6.5)
acetic acid		
43	169.4	-
44	22.4	1.82, s

Table S12. NMR data of 27. For atom numbering see Figure S59. * signals overlapping

malpinin-5-FAM (27)

pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])
tryptophan		
1	173.3	-
2	53.4	4.49, m*
2 NH	-	7.84, m*
3	26.8	3.21, m*
4	109.8	-
5	123.7	7.19, m*
5 NH	-	10.83, s
6	127.1	-
7	118.0	7.53, d (7.9)
8	118.3	6.98, t (7.4)
9	120.9 (1)	7.06, t (7.6)
10	111.3	7.33, d (8.2)
11	136.1	-
dehydrobutyrine		
12	164.3	-
13	130.2	-
13 NH	-	9.14, s
14	127.9	6.33, q (6.9)
15	13.4	1.55, d (6.9)
phenylalanine		
16	169.9	-
17	54.0	4.68, m*
17 NH	-	8.42, dd (8.0, 7.2)
18	36.8	2.83, dd (13.2, 11.1)
19	138.4	-
20, 24	130.6	7.46, d (8.3)
21, 23	119.4	7.81, d (8.2)
22	135.1	-
16	169.9	-
leucine		
25	172.0	-
26	51.04	4.22, m*
26 NH	-	7.71, m*
27	41.0	1.15
28	23.7	1.30, m*
29	22.7	0.66, m*
30	21.7	0.68, m*

pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])
arginine		
31	170.9	-
32	51.8	4.23, m*
32 NH	-	8.02, m*
33	28.5	1.25, m*
34	24.9	A: 1.47
		B: 1.40
35	40.4	3.04, m*
35 NH		7.44, m*
36	156.6	-
leucine		
37	172.4	-
38	51.14	4.21, m*
38 NH	-	7.99, m*
39	40.6	1.38, m*
40	40	24.1
41	21.5	0.80, m
42	22.9	0.84, m
acetic acid		
43	169.4	-
44	22.4	1.81, s
5-FAM		
1'	120.9 (2)	8.66, m*
2'	145.9	-
3'	35.0	4.66, d (5.0)
3' NH	-	9.44, m
4'	164.7	-
5'	126.4	-
6'	134.8	8.29, d (8.2)
7'	124.2	7.39, d (8.0)
8'	154.8	-
9'	168.1	-
10'	123.5	8.52, s
11'	135.9	-
11' OH	-	-
12'	83.2	-
13', 24'	109.0	
14', 23'	129.0	6.56, m*
15', 22'	102.3	6.69, t (2.2)
16', 21'	159.6	-
17', 20'	112.6	6.55, m*
18', 19'	151.8	-

Table S13. Organisms used in this study.

species	strain numbers	origin	reference
Escherichia coli	XL1 blue	Agilent	
	KRX	Promega	
	SoluBL21	Genlantis	
	BL21	NEB	
Mortierella alpina	ATCC32222	ATCC (American Type Culture Collection)	[26]
Mortierella amoeboidea	CBS889.72	JMRC (Jena Microbial Resource Collection)	[27]

Table S14. Oligonucleotides used in this study. Restriction sites are underlined. Primer efficiency with its correlation coefficient (R^2) is indicated for qPCR primers using gDNA of *M. alpina* as template.

name	sequence 5'-3'	target	purpose/restriction site	qPCR	
				efficiency /R^2	length (bp) cDNA/gDNA
oJMW001	CATCGATCTGGCCTACATGG	*gpdA*	qPCR housekeeping gene	0.98/0.999	80/229
oJMW002	CCACCTTGCCCTTGTAGC	*gpdA*	qPCR housekeeping gene	0.98/0.999	80/229
oJMW003	GTATGTGCAAGGCCGGTTTCG	*actB*	qPCR housekeeping gene	1.00/0.999	100/224
oJMW004	CCCATACCGACCATCACACC	*actB*	qPCR housekeeping gene	1.00/0.999	100/224
oJMW157	GGGTGCAGAAGCCAACAAAC	*nps2*	qPCR GOI (NRPS gene)	1.02/0.999	160/263
oJMW158	CTGGGACCATGTAGTCAGGC	*nps2*	qPCR GOI (NRPS gene)	1.02/0.999	160/263
oJMW175	TACGAGGAGCCAAGAGGTG	*nps3 (malA)*	qPCR GOI (NRPS gene)	0.98/0.999	99/99
oJMW176	GACAAAGAAGCCGTCGTTCC	*nps3 (malA)*	qPCR GOI (NRPS gene)	0.98/0.999	99/99
oJMW161	CTCCGACAGTGGTGCCAAG	*nps5*	qPCR GOI (NRPS gene)	0.95/0.998	170/291
oJMW162	CATATGCCGTGTCAAGACTGG	*nps5*	qPCR GOI (NRPS gene)	0.95/0.998	170/291
oJMW163	GTTAGTTTCGGGTCAGGCAAC	*orf35*	qPCR (transcription factor)	1.02/0.995	80/229
oJMW164	GGTAAACCTGGTCCCACTGC	*orf35*	qPCR (transcription factor)	1.02/0.995	80/229
oJMW285	CGCAGTTTGGTTCACGTC	*orf34*	qPCR (glucosaminyl transferase)	1.00/0.999	201/326
oJMW286	GGCACTTTAGCACATGGAGC	*orf34*	qPCR (glucosaminyl transferase)	1.00/0.999	201/326
oJMW165	CTCAAGCGCATCAAGAAACTCC	*orf32*	qPCR (dehydratase)	0.97/0.998	167/167
oJMW166	GTTTGCGTCCTGATTCACCC	*orf32*	qPCR (dehydratase)	0.97/0.998	167/167
oJMW173	GATTGCTAGACGCCTCTGCAG	*orf29*	qPCR (amidase)	0.99/0.998	154/154
oJMW174	GCAGAGCTGTCTCCGGAAAC	*orf29*	qPCR (amidase)	0.99/0.998	154/154
oJMW169	CGGATACATACACATCTGGCGG	*orf27*	qPCR (protein kinase)	1.00/0.999	122/251
oJMW170	CCGATACCATCTCCAGTGCATC	*orf27*	qPCR (protein kinase)	1.00/0.999	122/251
oITS1	TCCGTAGGTGAACCTGCGG	ITS	strain identification		
oITS4	TCCTCCGCTTATTGATATGC	ITS	strain identification		
oMG270	GTGGTGGTGCTCGAGGATGGCACGGTGCTGTCCGAT	*malA* module 1	amplification		
oMG285	AGGAGATATACCATGATGAACAACATTGACCATC	*malA* module 1	amplification		
oMG283	CATGGTATATCTCCTTCTTAAAG	pET28a vector	vector amplification		
oMG284	CTCGAGCACCACCACCACCACC	pET28a vector	vector amplification		
oJMW079	GATCACATTGTCGAGCAAGTGC	*malA* module 2	amplification		
oJMW080	GACATTCGGTGGCACCACAATG	*malA* module 2	amplification		
oJMW081	ATATATGCTAGCGATCACATTGTCGAGCAAGTGC	*malA* module 2	vector cloning (*Nhe*I)		
oJMW153	ATATATGCGGCCGCGACATTCGGTGGCACCACAATG	*malA* module 2	vector cloning (*Not*I)		
oJMW149	GTCGACCAAATCCCAGGAGG	*malA* module 3	amplification		
oJMW150	CGTGATGACGTTCGATGGCAC	*malA* module 3	amplification		
oJMW151	ATATATGCTAGCGTCGACCAAATCCCAGGAGG	*malA* module 3	vector cloning (*Nhe*I)		
oJMW152	ATATATAAGCTTCGTGATGACGTTCGATGGCAC	*malA* module 3	vector cloning (*Hind*III)		
oIW021	GCTCTCGCTCAGTCCATCG	*malA* module 4	amplification		
oIW022	CTCTTCCTGTGCCAACTGTTC	*malA* module 4	amplification		
oIW023	TATATAGGCTAGCGCTCTCGCTCAGTCCATCG	*malA* module 4	vector cloning (*Nhe*I)		
oIW024	ATATATGCGGCCGCTACTCTTCCTGTGCCAACTGTT	*malA* module 4	vector cloning (*Not*I)		
oJMW141	ATGGCCATTACACCCGTCTC	*malA* module 5	amplification		
oJMW142	AGGTATGACAATGGCTTGATGGT	*malA* module 5	amplification		
oBWma01	ATATATGCTAGCATGGCCATTACACCCGTCTCGA	*malA* module 5	vector cloning (*Nhe*I)		
oBWma02	ATATATAAGCTTAGGTATGACAATGGCTTGATGGTGTC	*malA* module 5	vector cloning (*Hind*III)		
oJMW145	GCCGCATCTATCGGACACC	*malA* module 6	amplification		
oJMW146	CGGAGTGATGTAGTTCGGAGG	*malA* module 6	amplification		
oJMW147	ATATATGCTAGCGCCGCATCTATCGGACACC	*malA* module 6	vector cloning (*Nhe*I)		
oJMW148	CTCTCTAAGCTTCGGAGTGATGTAGTTCGGAGG	*malA* module 6	vector cloning (*Hind*III)		
oJMW143	GTTCCTGGCGGAGTTGCC	*malA* module 7	Amplification		
oJMW144	ATCGTAGGACTCGTCCTGTGTC	*malA* module 7	Amplification		
oBWma03	ATATATCATATGGTTCCTGGCGGAGTTGCCAAC	*malA* module 7	vector cloning (*Nde*I)		
oBWma04	ATATATAAGCTTATCGTAGGACTCGTCCTGTGTCA	*malA* module 7	vector cloning (*Hind*III)		

Table S15. Plasmids used in this study.

plasmid	vector backbone	purpose	gene product	source/reference
pJET1.2	-	Amplification of DNA	-	ThermoFisher
pET28a (+)	-	expression vector	-	Agilent
pMG027	pET28a (+)	expression of *malA*-M1	MalA module 1	This study.
pJMW31	pET28a (+)	expression of *malA*-M2	MalA module 2	This study.
pJMW33	pET28a (+)	expression of *malA*-M3	MalA module 3	This study.
pJMW10	pET28a (+)	expression of *malA*-M4	MalA module 4	This study.
pJMW24	pET28a (+)	expression of *malA*-M5	MalA module 5	This study.
pJMW27	pET28a (+)	expression of *malA*-M6	MalA module 6	This study.
pJMW26	pET28a (+)	expression of *malA*-M7	MalA module 7	This study.

Table S16. HPLC methods used in this study.

	method A	method B	method C	method D
instrument	Waters ACQUITY H-class UPLC	Agilent 1290 infinity II UHPLC	Agilent 1290 infinity II UHPLC	Agilent 1290 infinity II UHPLC
solvent A	water + 0.1% FA	water + 0.1% FA	water + 0.1% FA	water + 0.1% FA
solvent B	acetonitrile	acetonitrile	acetonitrile	acetonitrile
gradient	0-4 min: 10-50% A, 5-9 min: 50-10% A	0-4 min: 5-72% B, 4-4.5 min: 72-95% B, 4.5-5 min: 95% B	0-10 min: 1-40% B, 10-12 min: 40-95% B, 12-13 min: 95% B, 13-14 min 95-1% B	0-10 min: 1-50% B, 10-11 min: 50-100% B
temperature	30°C	30°C	30°C	30°C
flow	0.4 mL min^{-1}	1 mL min^{-1}	1 mL min-1	1 mL min-1
column	ACQUITY UPLC BEH Amide column	Zorbax Eclipse Plus C18 RRHD (Agilent)	Zorbax Eclipse Plus C18 RRHD (Agilent)	Luna Omega polar C18 column (Phenomenex)
column dimension	50 mm × 2.1 mm, 1.7 µm	50 mm × 2.1 mm, 1.8 µm	50 mm × 2.1 mm, 1.8 µm	50 mm × 2.1 mm, 1.6 µm
detection	Waters Xevo TQ-S micro tandem quadrupole instrument	Agilent 6130 single quadrupole MS, ESI ionization source	Agilent 6130 single quadrupole MS, ESI ionization source	Agilent 6130 single quadrupole MS, ESI ionization source

	method E	method F	method G	method H
instrument	Preparative HPLC Agilent 1260	Preparative HPLC Agilent 1200	Preparative HPLC Agilent 1200	Preparative HPLC Agilent 1200
solvent A	water + 0.1% TFA	water + 0.1% TFA	water + 0.1% TFA	water + 0.1% TFA
solvent B	acetonitrile	acetonitrile	methanol	acetonitrile
gradient	0-20 min: 10-100% B, 20-27 min: 100% B	0-0.5 min: 35% B, 0.5-12.5 min: 35-65% B, 12.5-13 min: 65-100% B, 13-14 min: 100% B	0-0.5 min: 70% B, 0.5-15 min: 70-100% B, 15-16 min: 100% B	0-9.5 min: 45% B, 9-9.5 min: 45-100% B, 9.5-10 min 100% B
temperature	25°C	12°C	12°C	40°C
flow	20 mL min-1	2.5 mL min^{-1}	2 mL min^{-1}	2 mL min^{-1}
column	Luna C18 (Phenomenex)	Zorbax Eclipse XDB-C18 (Agilent)	SynergiHydro-RP 80 Å (Phenomenex)	Zorbax Rx-C18 (Agilent)
column dimension	250 mm × 21.2 mm, 10 µm	250 mm × 9.4 mm, 5 µm	250 × 10 mm, 4 µm	250 x 9.4 mm, 5 µm
detection	DAD, λ = 254 nm	DAD, λ = 254 nm	DAD, λ = 254 nm	DAD, λ = 225 nm

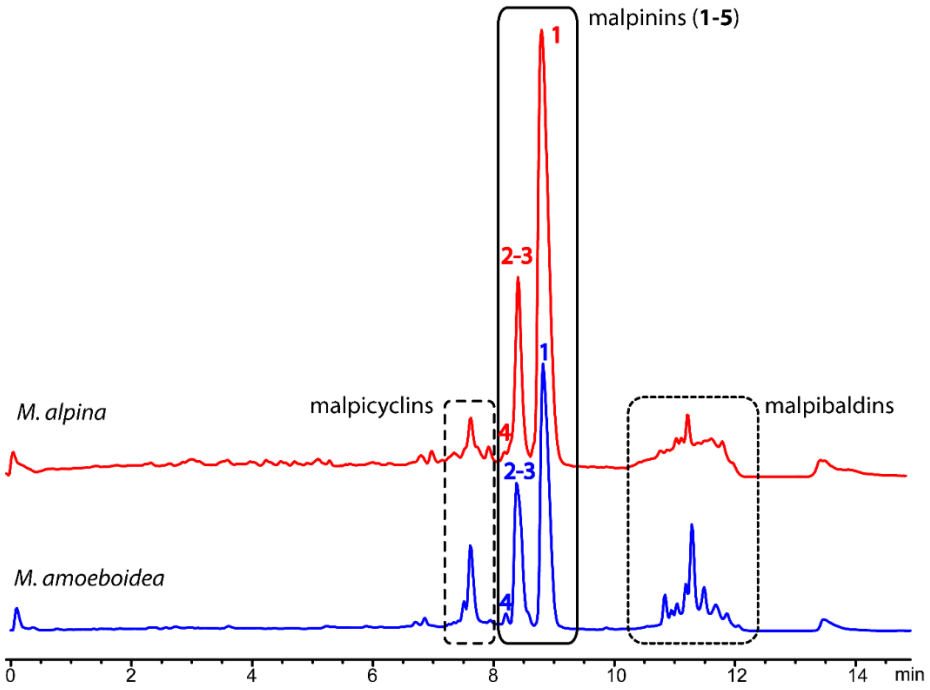


Figure S1. Total ion chromatograms of crude metabolite extracts from *M. alpina* and *M. amoeboidea*. The three major series of peptides, malpinins (**1-5**, box with continuous line), malpibaldins (dotted line) and malpicyclins (dashed line), are produced in both strains in varying amounts. Total ion counts were monitored from *m/z* 100 to 1600 (positive mode). Compound **5** is produced in traces (not shown).

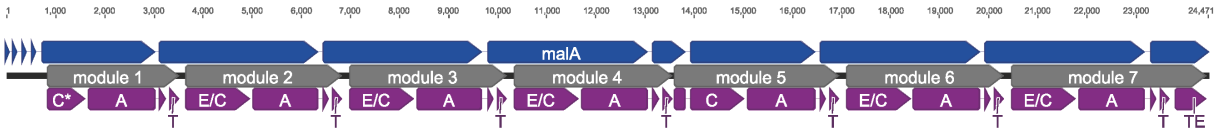
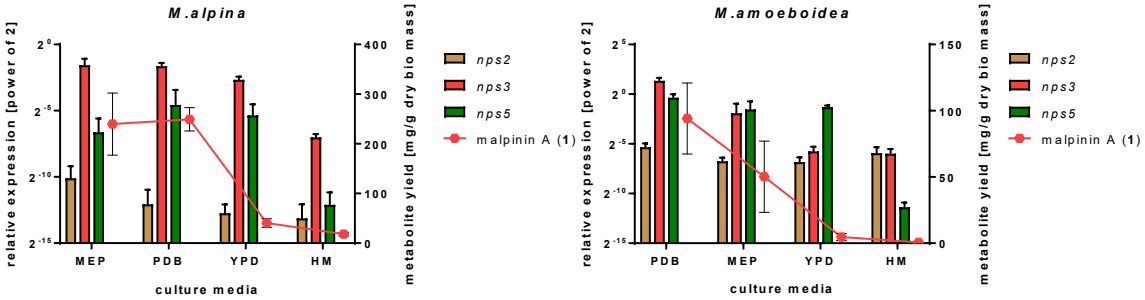


Figure S2. Schematic representation of the *malA* (*nps3*) gene. The intron/exon pattern is indicated by blue arrows (first row). Exons are boxed. Note, that all exons encoding A domains contain one intron each at near identical positions. The individual modules are highlighted in grey (second row). The required enzyme domains are indicated in purple (lowest row). Note, that the first C starter domain is truncated and inactive (asterisk). A, adenylation domain; C, condensation domain; E/C, dual epimerization/condensation domain; T, thiolation domain; TE, thioesterase.



strain	candidate NRPS	Pearson R	P value
M. alpina	*nps2*	0.657	0.343
	nps3 (malA)	**0.943**	**0.057**
	nps5	0.499	0.501
M. amoeboidea	*nps2*	0.649	0.351
	nps3 (malA)	**0.904**	**0.096**
	nps5	0.838	0.162

Figure S3. Expression analysis of candidate *nps* genes in *M. alpina* and *M. amoeboidea*. qRT-PCR analysis was carried out using the actin gene (*actA*) and glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) as internal reference gene.[19] Fold expression of candidate genes *nps2*, *nps3* (*malA*) and *nps5* (left ordinate) and malpinin A yields per fungal biomass (right ordinate) are presented for *M. alpina* (left panel) and *M. amoeboidea* (right panel). A strong linear correlation between gene expression and malpinin production was determined for *malA* in both strains (Pearson´s Correlation Coefficient R > 0.90). MEP, malt extract - peptone; PDB, potato dextrose broth; YPD, yeast extract - peptone - dextrose; HM, hay medium.

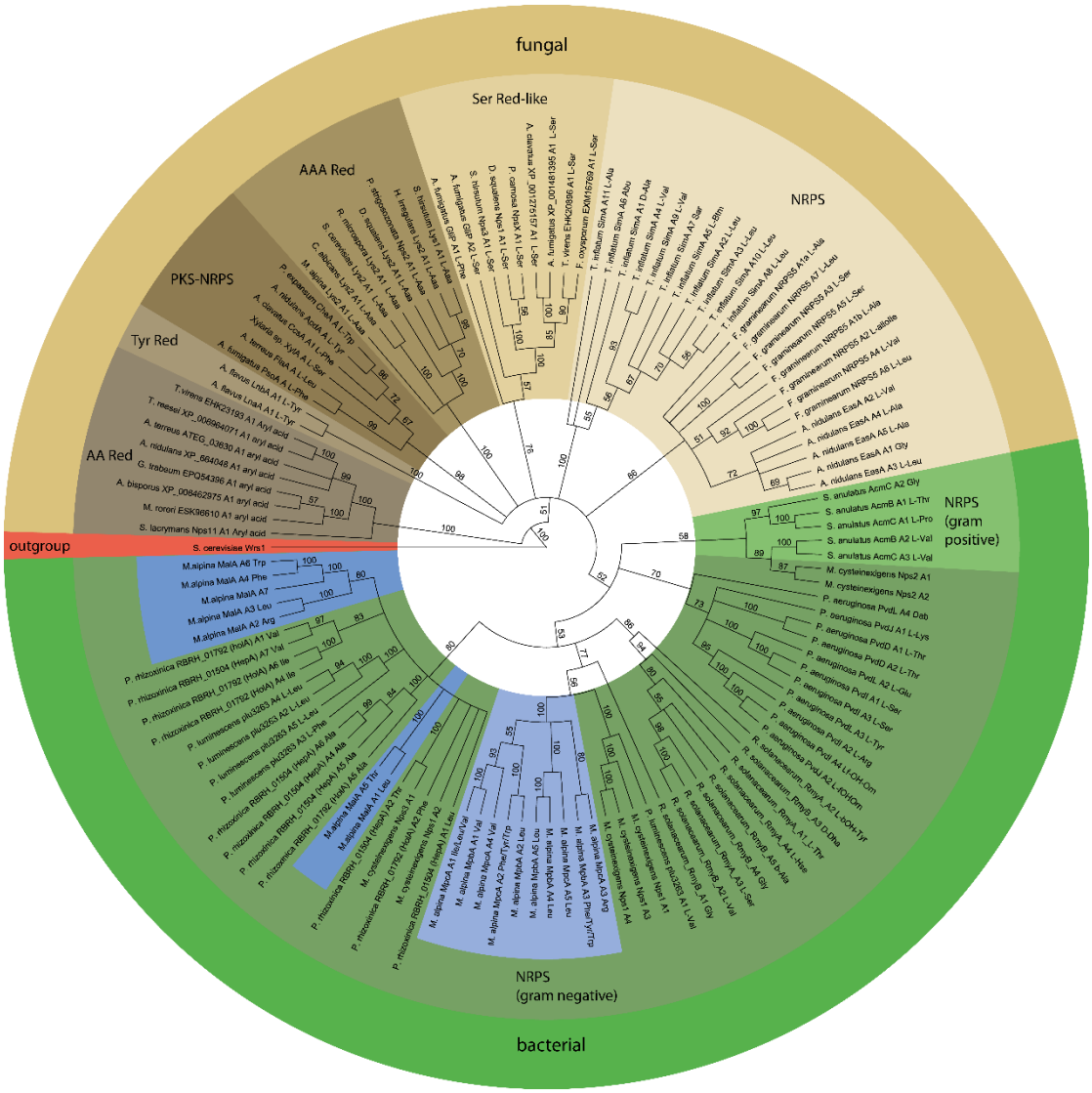


Figure S4. Phylogenetic analysis of A domains from *M. alpina* and other fungal or bacterial representatives. A set of 123 A domains were extracted from NRPSs, NRPS-like proteins, and PKS/NRPS hybrids from *M. alpina* (blue), (endo)bacteria (green), and higher fungi (taupe/amber) as described previously.[19] The A domain of the cytoplasmatic tryptophanyl-tRNA synthetase Wrs1 from *Saccharomyces cerevisiae* served as the outgroup (red). The A domains from *M. alpina* - including A domains from MalA - cluster together with bacterial counterparts. The percentual bootstrap support is indicated. AAA red, α-2-aminoadipate reductase; AA Red, aryl acid reductase; NRPS, nonribosomal peptide synthetase; PKS-NRPS, polyketide synthase-nonribosomal peptide synthetase hybrid; Ser Red, serine reductase; Tyr Red, tyrosine reductase.

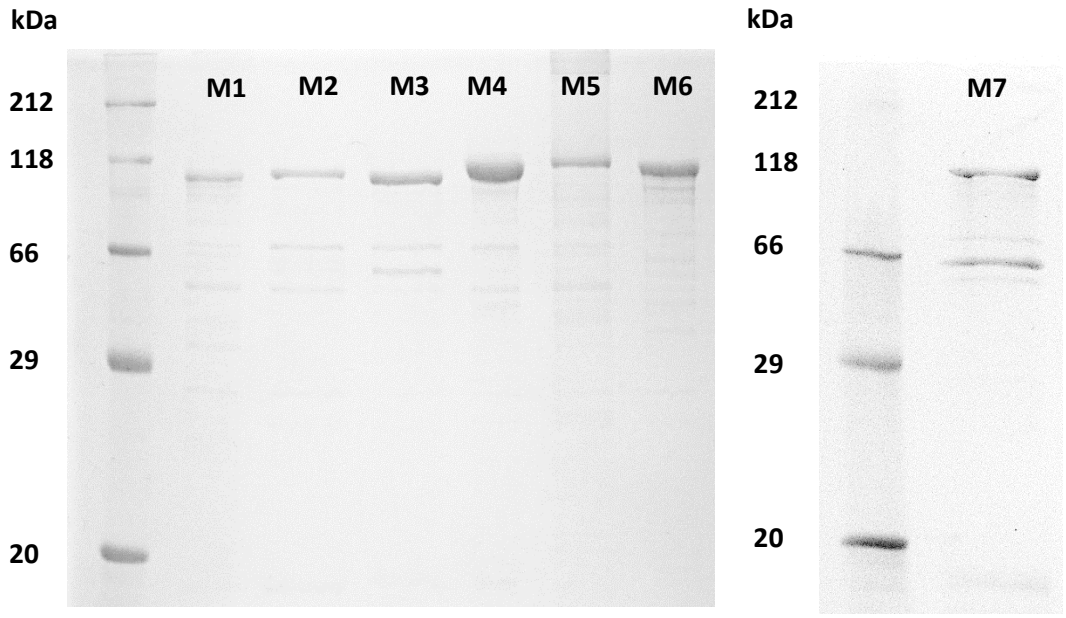


Figure S5. SDS polyacrylamide gel electrophoresis of MalA modules 1-7. A total of 1-3 µg biHis$_6$-tagged enzyme are loaded per lane. The expected molecular weights are: M1, 110.9 kDa; M2, 120.1 kDa; M3, 119.6 kDa; M4, 123.8 kDa; M5, 123.1 kDa; M6, 125.5 kDa; and M7, 120.2 kDa.

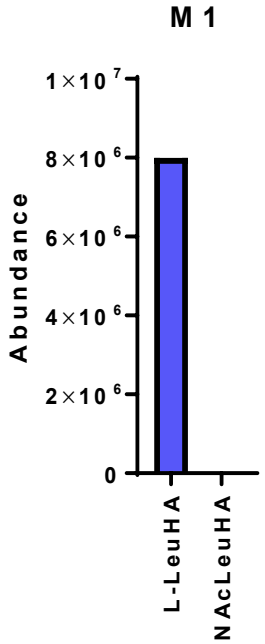


Figure S6. HAMA analysis of MalA-M1 with L-leucine and *N*-acetyl-L-leucine as substrates. L-leucine was successfully converted to L-leucine-hydroxamate (L-LeuHA) by MalA-M1. In contrast, the corresponding *N*-acetyl-L-leucine-hydroxamate (NAcLeuHA) was not determined when *N*-acetyl-L-leucine was applied.

Figure S7. Sequence alignment of the MalA starter C (C_s) domain and other experimentally characterised starter C domains. Sequence alignment of N-terminal C starter domains (C_s) are shown for: AMYAL_RS0130210,[28] ErcD,[29] Hep,[30] ArfA,[31] SyfA (Accession: EPF66764)[32], JesA,[33] and MalA (this study). The canonical condensation domain C3 of the gramicidin S synthetase GrsB[34] served as reference to identify the seven core motifs (C1- C7).[35] With the exception of MalA C_s and ACV2 C_s, a conserved double histidine motif is evident (C3 motif). C domains were obtained from proteins from bacteria (*Brevibacillus brevis* (syn. *Aneurinibacillus migulanus*), *Amycolatopsis alba, Saccharopolyspora erythraea, Mycetohabitans rhizoxinica, Pseudomonas syringae* and two additional *Pseudomonas* spec.) and fungi (*Aspergillus flavus*, *Tolypocladium inflatum*, *Mortierella alpina* and *Penicillium rubens*).

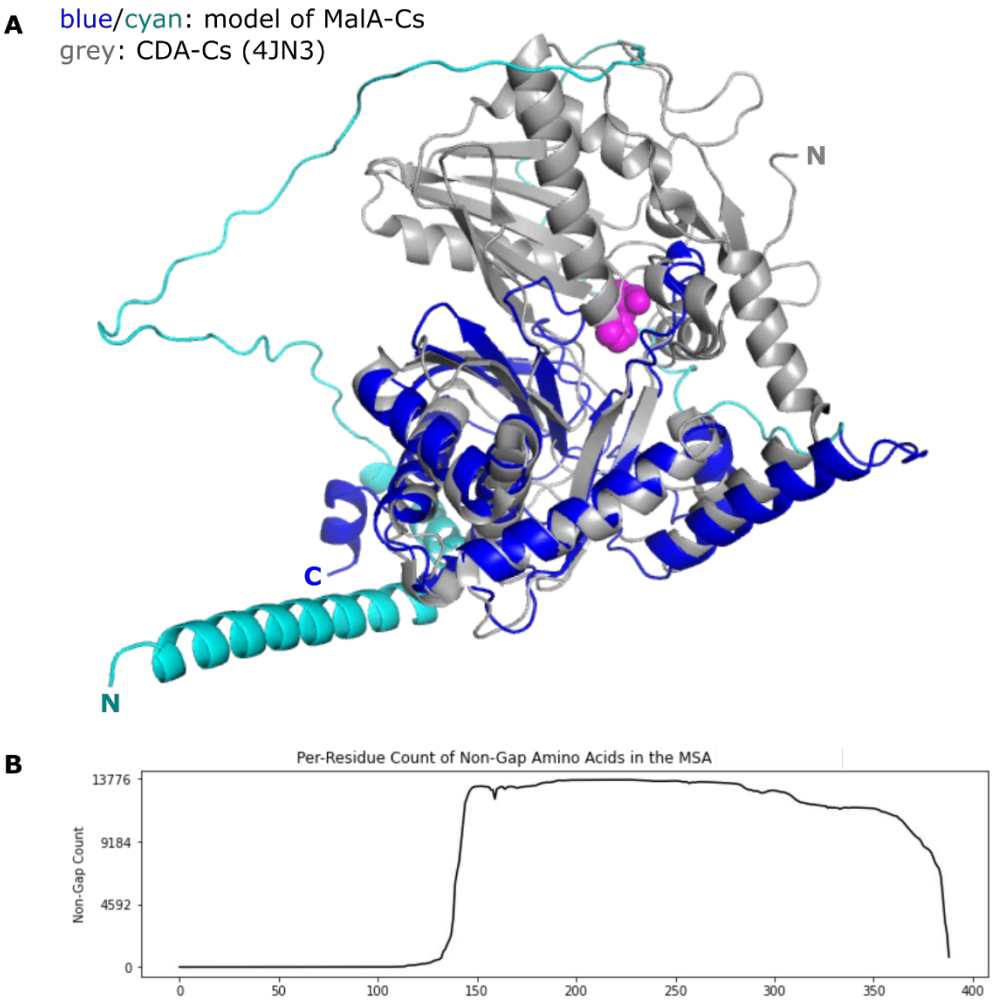


Figure S8. **A**. Structural model of MalA-C_s. The N-terminal domain of MalA, which shows partial homology to Cs domains, has been structurally modelled with AlphaFold v2.1.0[20] using a publicly available script on Google Colab (AlphaFold.ipynb). The model has been superimposed with a crystal structure of the C_s domain from CDA biosynthesis (PDB 4JN3, grey cartoon) in Pymol v2.2.2 using the "super" command. Only the C-terminal part of the model (blue cartoon) shows sequence homology and aligns with the C_s domain structure (RMSD = 1.632 Å for residues 129-389). The N-terminal lobe of the C_s-domain carrying the important catalytic His residue (His157, spheres in magenta) cannot be modelled in the N-terminal MalA domain (cartoon in cyan) because a multiple sequence alignment (**B**) for this part of the protein could not be obtained. Therefore, the N-terminal domain of MalA is highly unlikely to be a catalytically active acyl transferase.

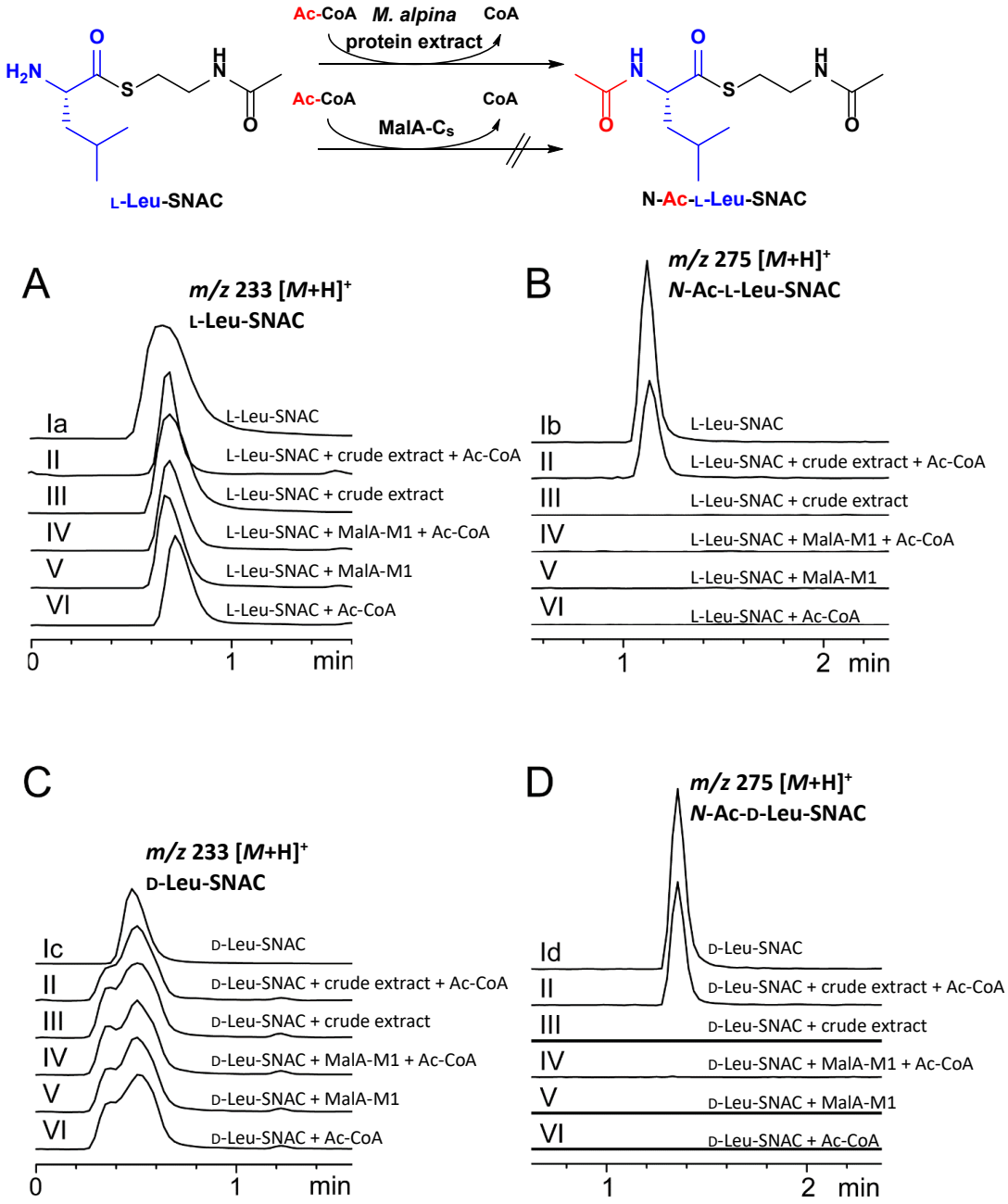
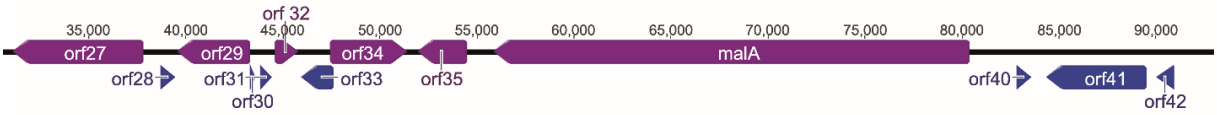


Figure S9. Chromatographic analysis of *N*-acetylation of leucyl-SNAC thioesters. *M. alpina* protein crude extracts or purified MalA-M1 was incubated with L-Leu-SNAC (**A, B**) or D-Leu-SNAC (**C, D**) in presence and absence of Ac-CoA. Extracted ion chromatograms (EIC) were recorded for L- and D-leucyl-SNAC at *m/z* 233 (**A** and **C**) and for their respective acetyl amides, L- and D-*N*-acetyl-leucyl-SNAC at *m/z* 275 (**B** and **D**). The traces are: I) respective synthetic standard (Ia L-Leu-SNAC; Ib N-acetyl-L-Leu-SNAC, Ic D-Leu-SNAC, Id N-acetyl-D-Leu-SNAC), II) *M. alpina* crude protein extract with substrate and Ac-CoA, III) *M. alpina* crude protein extract with substrate, but without Ac-CoA, IV) purified MalA-M1 with substrate and Ac-CoA; V) purified MalA-M1 with substrate, but without Ac-CoA, VI) substrates with Ac-CoA without enzyme. Note, that acetylation of both L- and D-Leu-SNAC is detectable with *M. alpina* protein crude extract, but not with MalA-C_s.

A



			Pearson´s correlation coefficient R (*p* value)						
	location on contig tig00000075	function	orf27	orf29	orf32	orf34	orf35	malA	malpinin A (**1**)
orf27	37833 -> 31089	protein kinase	-	0.906 (0.094)	0.265 (0.735)	0.155 (0.845)	0.161 (0.839)	-0.448 (0.552)	-0.714 (0.286)
orf29	40822 -> 39567	amidase		-	0.605 (0.395)	0.546 (0.454)	-0.227 (0.773)	-0.197 (0.803)	-0.509 (0.491)
orf32	44581 -> 45830	epimerase/dehydratase			-	0.764 (0.236)	-0.522 (0.478)	0.629 (0.371)	0.359 (0.641)
orf34	47439 -> 51400	N-Ac-glucosaminyl transferase				-	-0.925 (0.075)	0.226 (0.774)	0.052 (0.948)
orf35	54490 -> 51959	transcription factor					-	-0.174 (0.826)	-0.124 (0.876)
malA	80366 -> 55894	NRPS						-	**0.943 (0.057)**

B

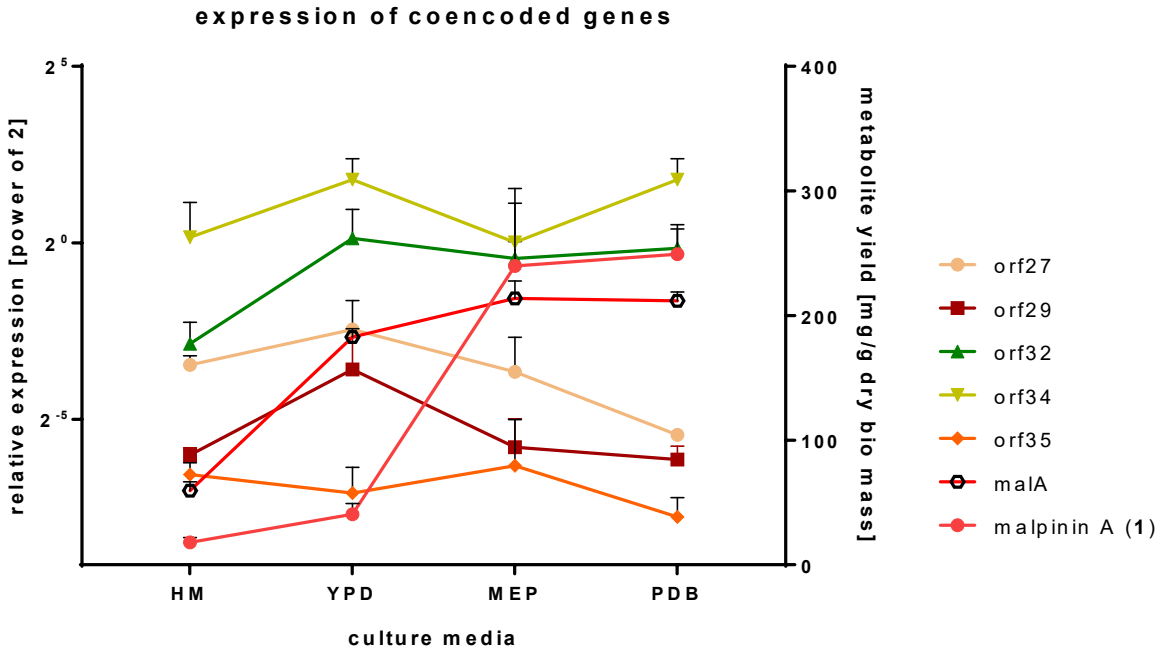


Figure S10. Bioinformatic and expression analysis of genes encoded adjacent to *malA*. A. Potential malpinin biosynthesis gene cluster**.** Open reading frames were predicted using Augustus[36] plugin for Geneious 10.2.4. The genome of *Rhizopus oryzae* served as reference. Genes with distinct predicted function (purple) are listed in the table. Open reading frames with unknown function are highlighted in blue. **B.** Expression analysis of genes adjacent to *malA*. Expression values of the genes (left y-axis), and metabolite production rate (right y-axis) are indicated. qRT-PCR analysis was carried out using the actin gene (*actA*) and glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) as internal reference gene. A strong linear correlation (R > 0.94) between gene expression and malpinin A production was evident for *malA,* but for none of the remaining genes. Pearson´s Correlation Coefficient R and according *p* values are given in the table. MEP, malt extract – peptone medium; PDB, potato dextrose broth; YPD, yeast extract - peptone – dextrose medium; HM, hay medium.

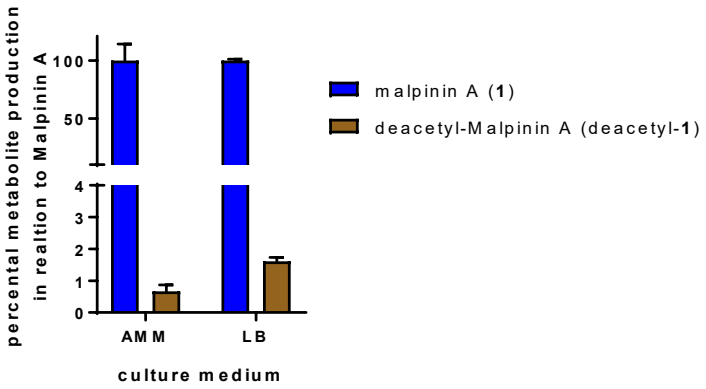


Figure S11. Production of deacetyl-1 in AMM and LB medium. Yields are given as ratios in relation to **1.**

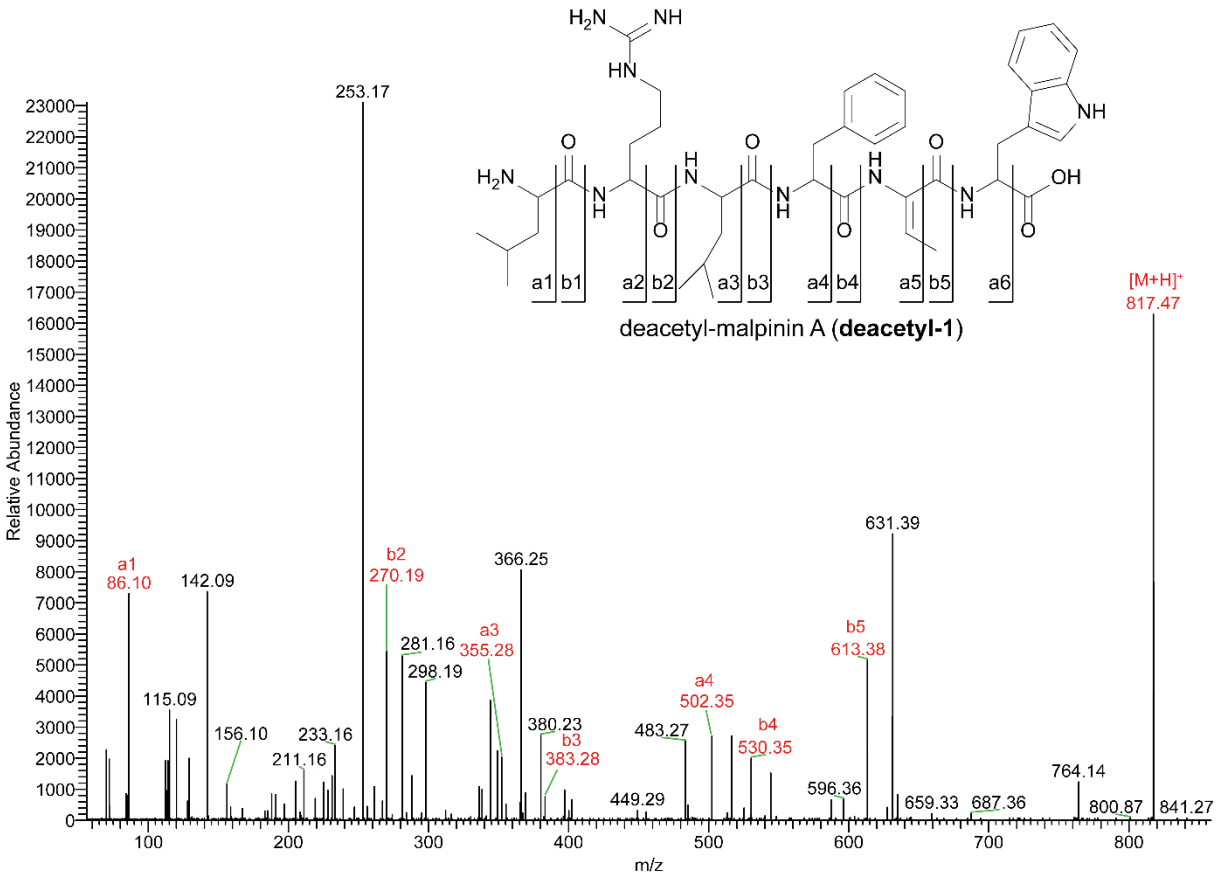


Figure S12. HR-ESI-MS/MS spectra of deacetyl-1. Spectra were obtained using 30% collision energy.

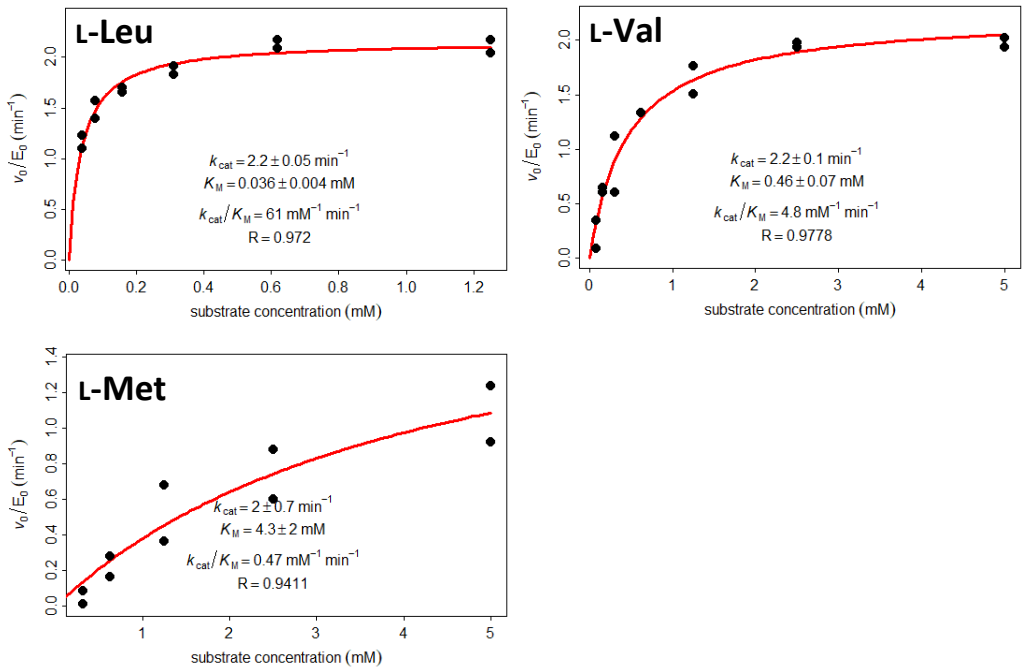


Figure S13. Michaelis-Menten kinetics of MalA-M3 for different substrates. MalA-M3 was incubated with various concentrations (0.1 – 5 mM) of substrate amino acids (L-leucine, L-valine and L-methionine) and turnover was determined by the MesG assay.

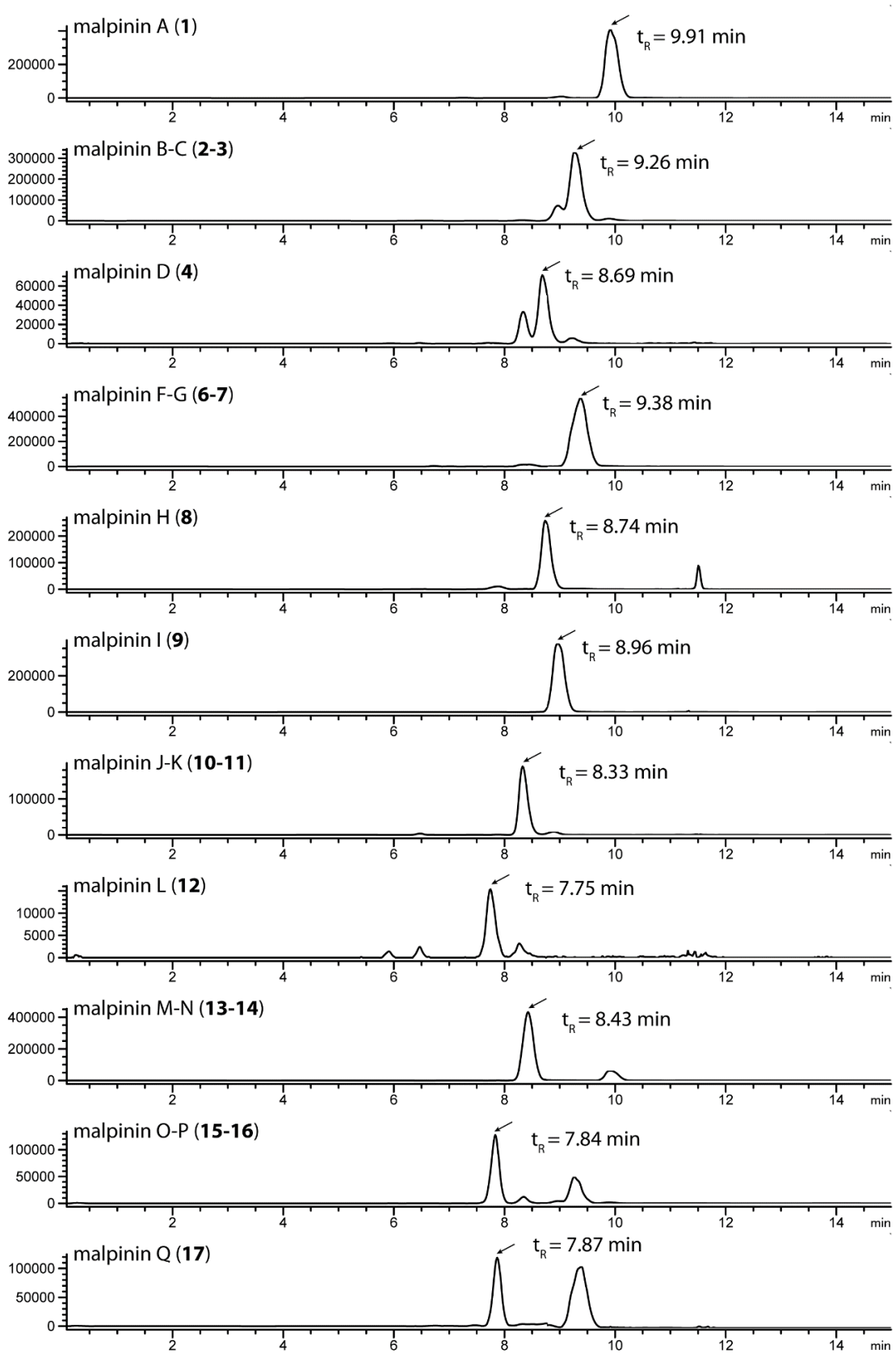


Figure S14. Extracted ion chromatograms (EICs) of metabolite extracts from cultures supplemented with L-methionine, recorded by UHPLC method C (Table S16) for better resolution and to inhibit coelution of metabolites.

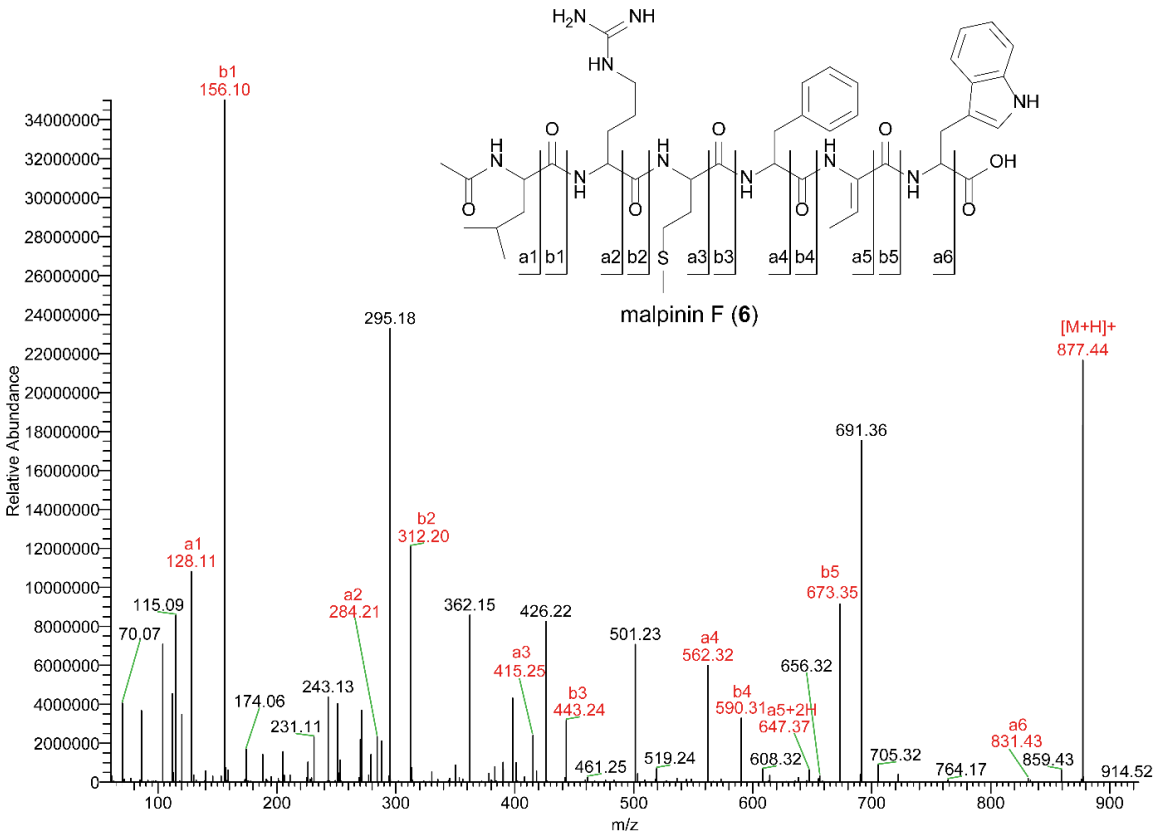


Figure S15. HR-ESI-MS/MS spectrum of 6.

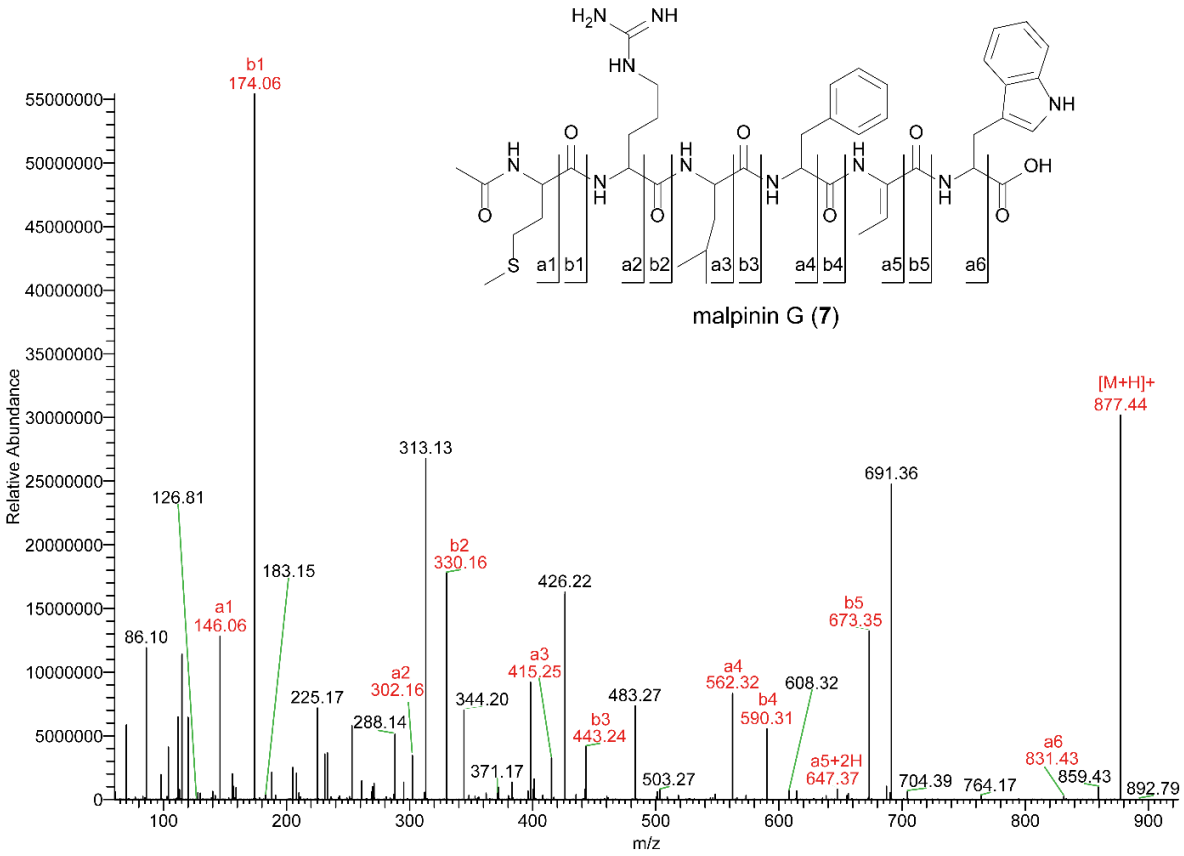


Figure S16. HR-ESI-MS/MS spectrum of 7.

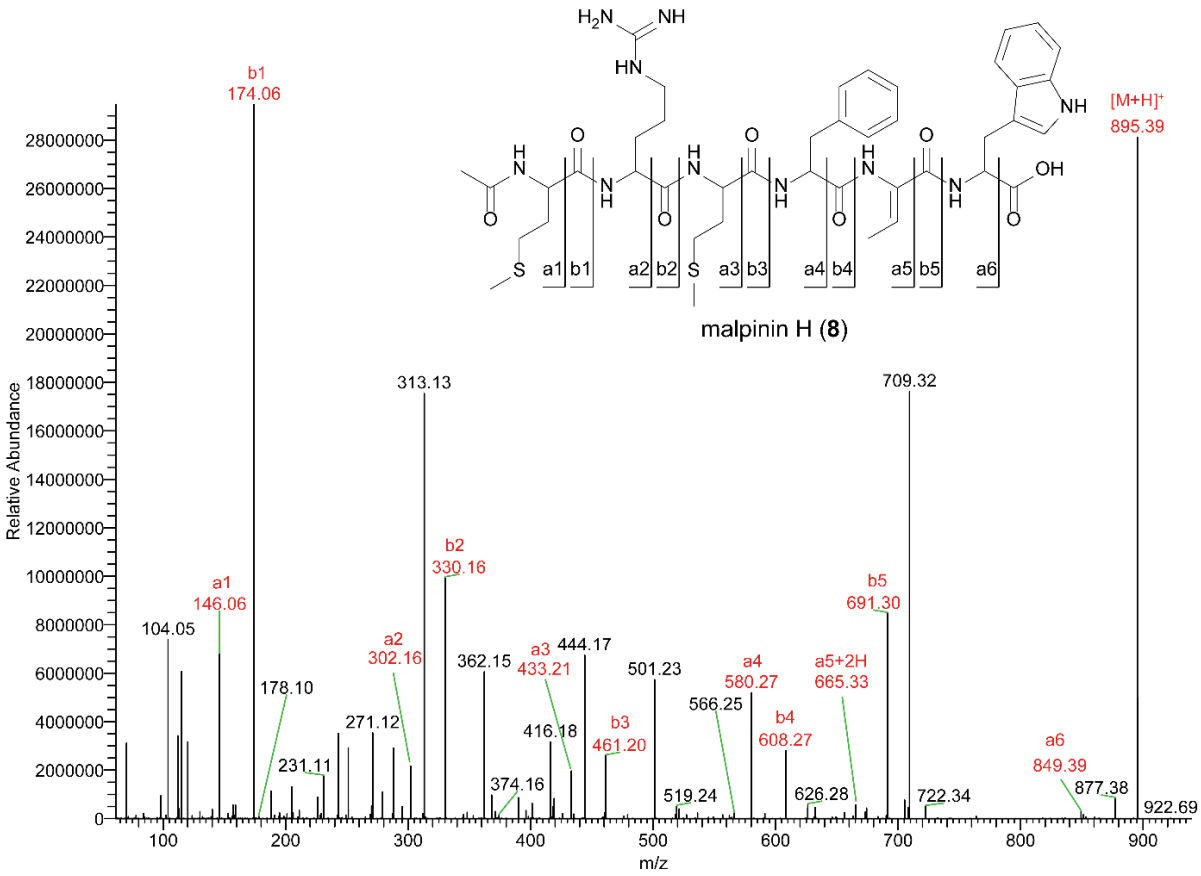


Figure S17. HR-ESI-MS/MS spectrum of 8.

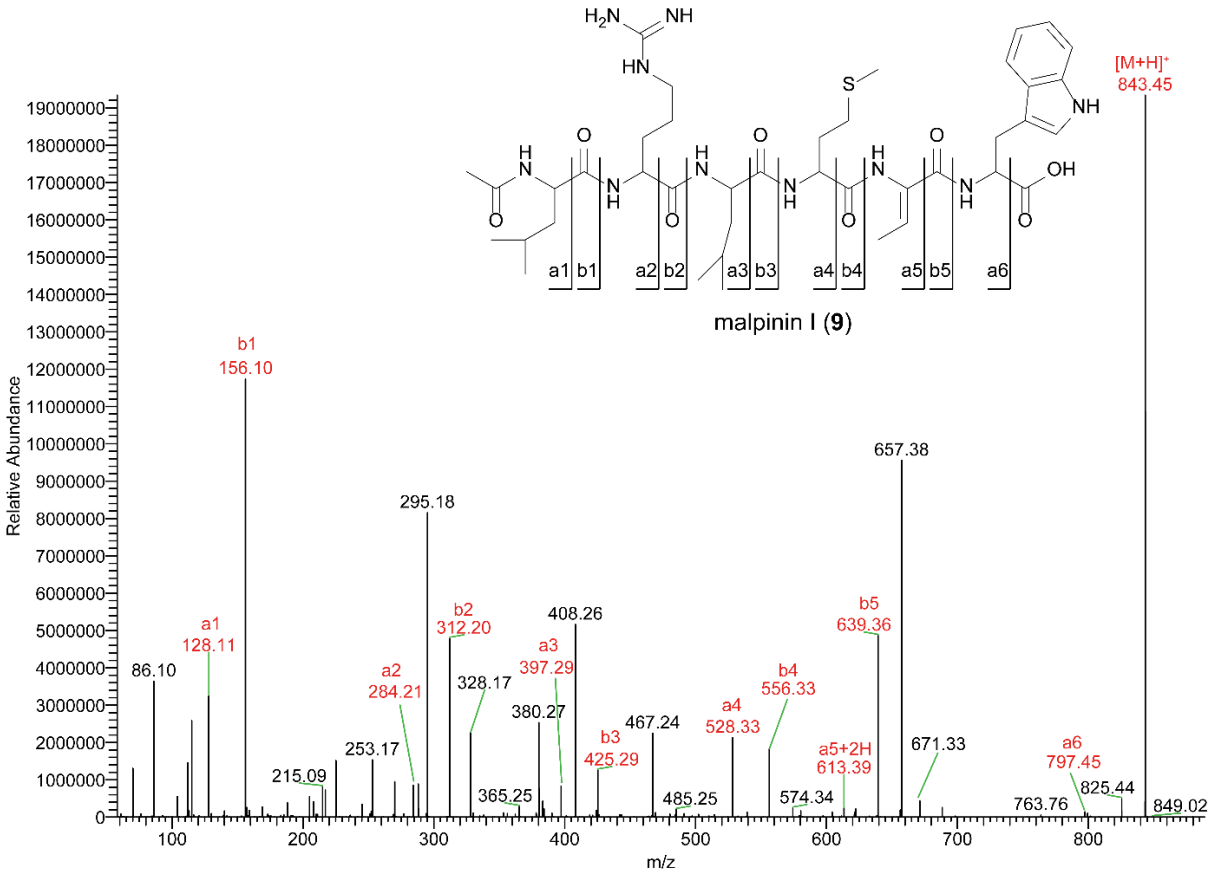


Figure S18. HR-ESI-MS/MS spectrum of 9.

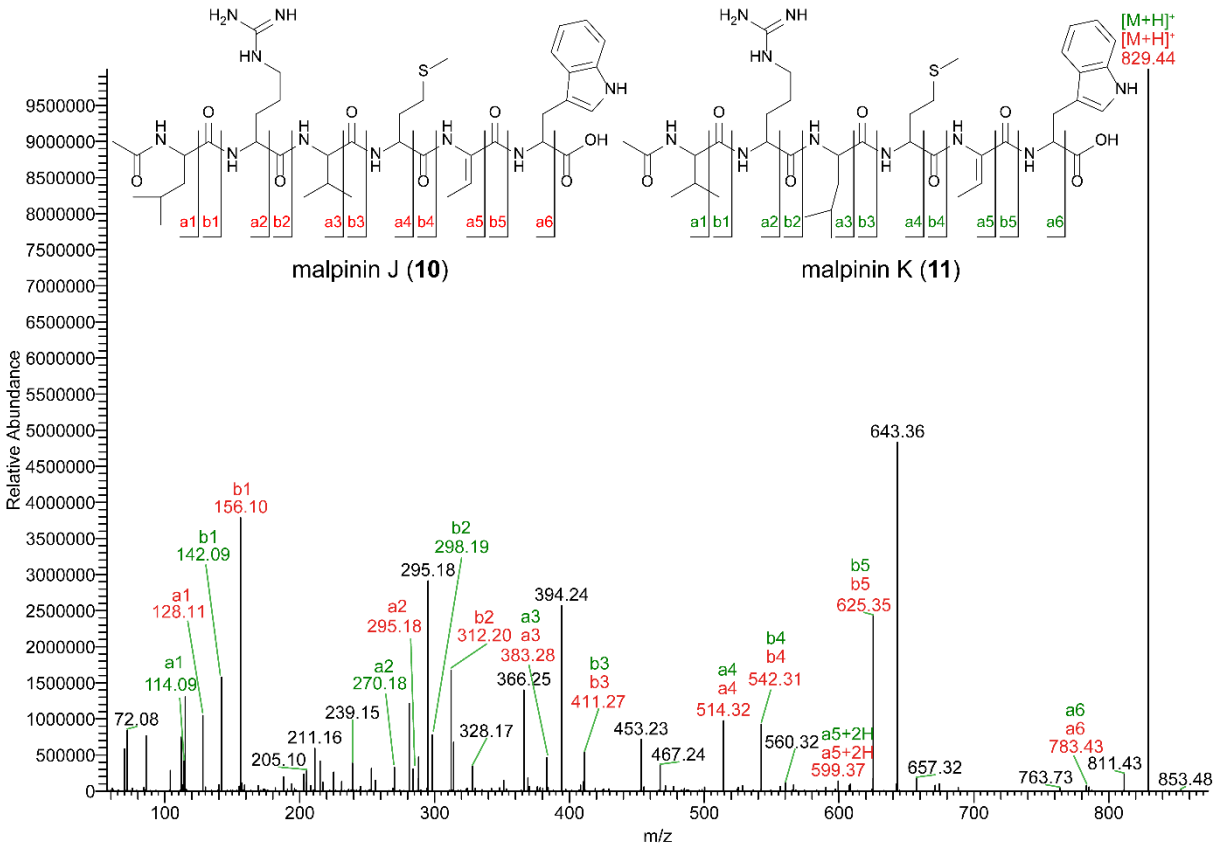


Figure S19. HR-ESI-MS/MS spectrum of the two coeluting isomers 10 and 11.

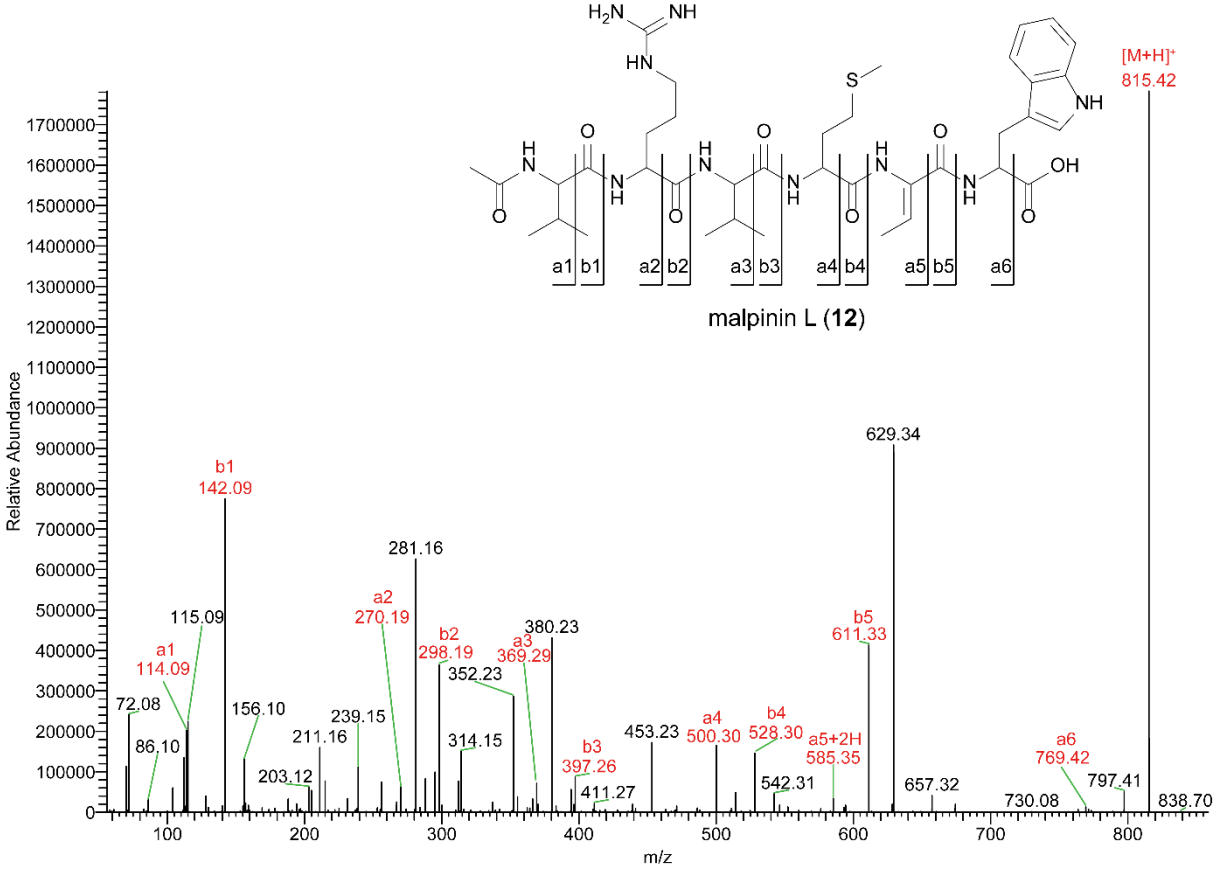


Figure S20. HR-ESI-MS/MS spectrum of 12.

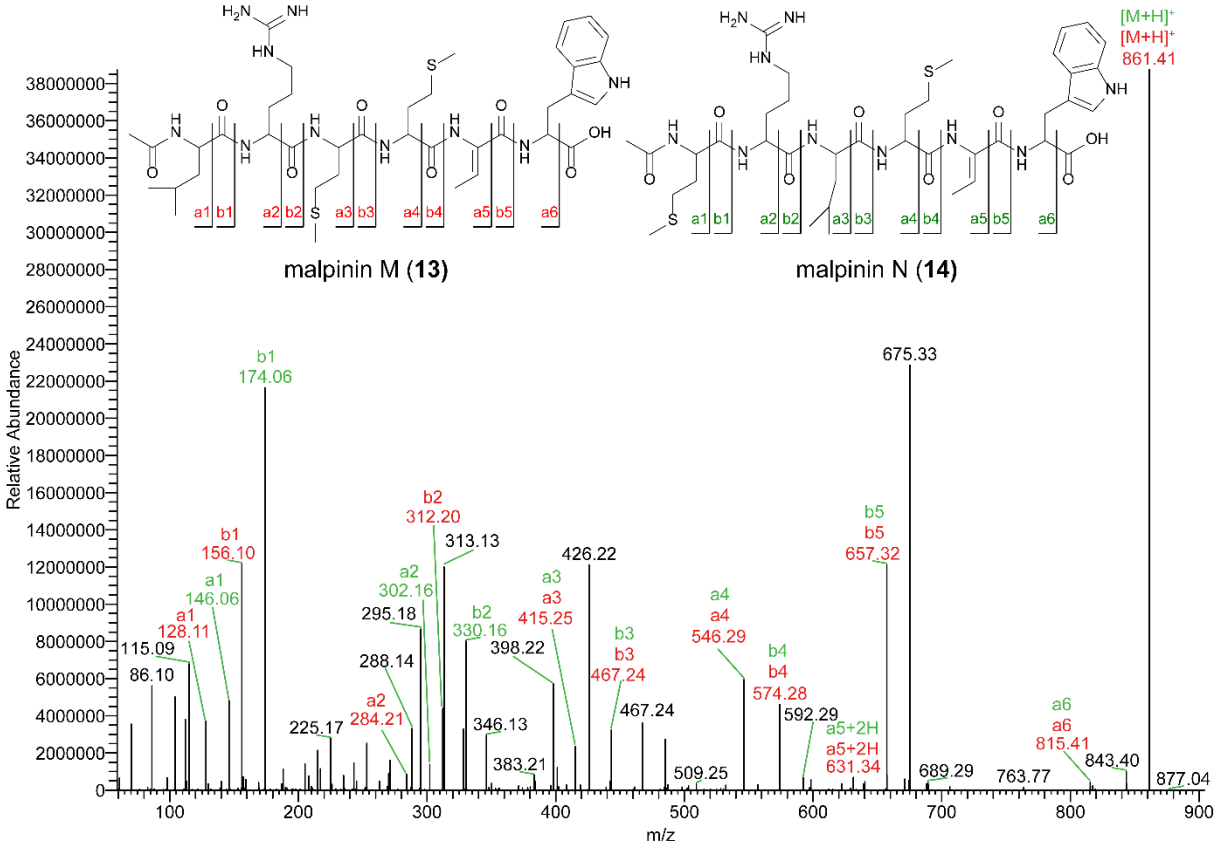


Figure S21. HR-ESI-MS/MS spectrum of the two coeluting isomers 13 and 14.

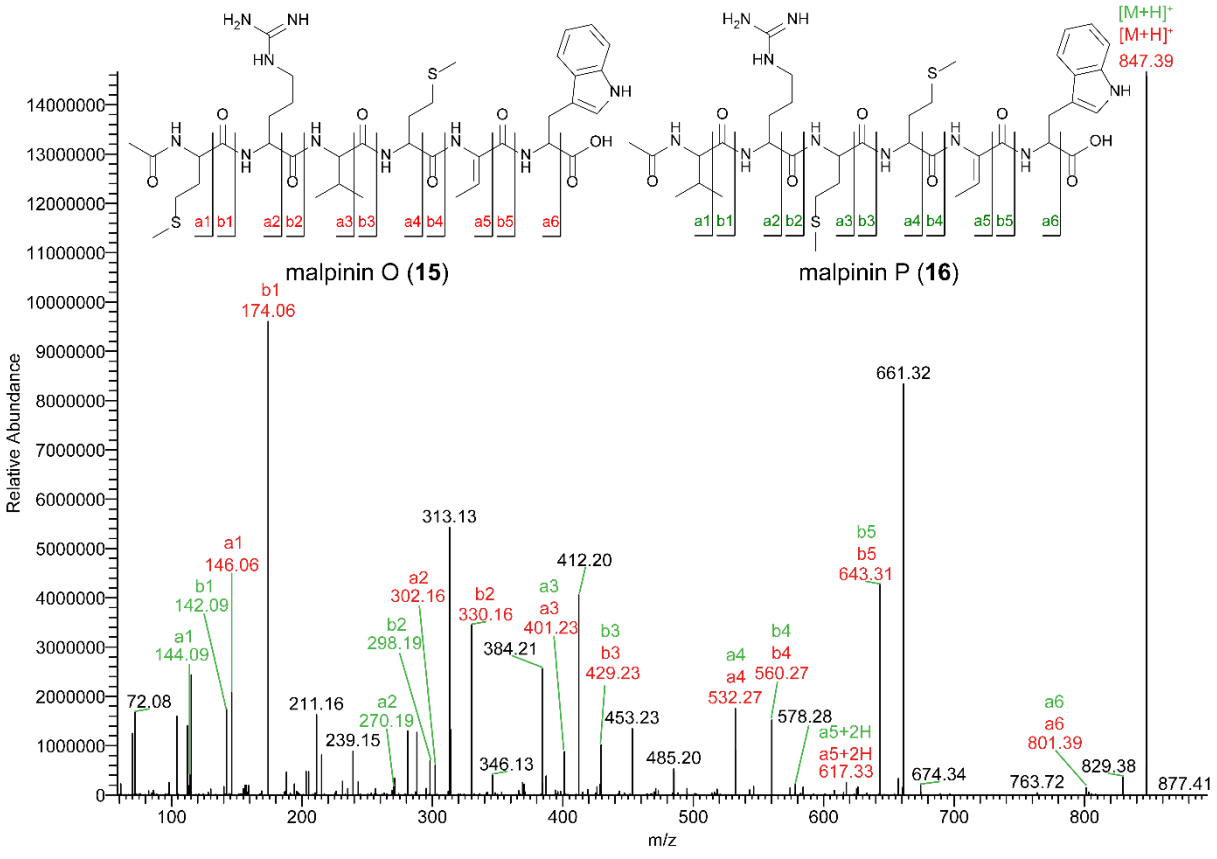


Figure S22. HR-ESI-MS/MS spectra of the two coeluting isomers 15 and 16.

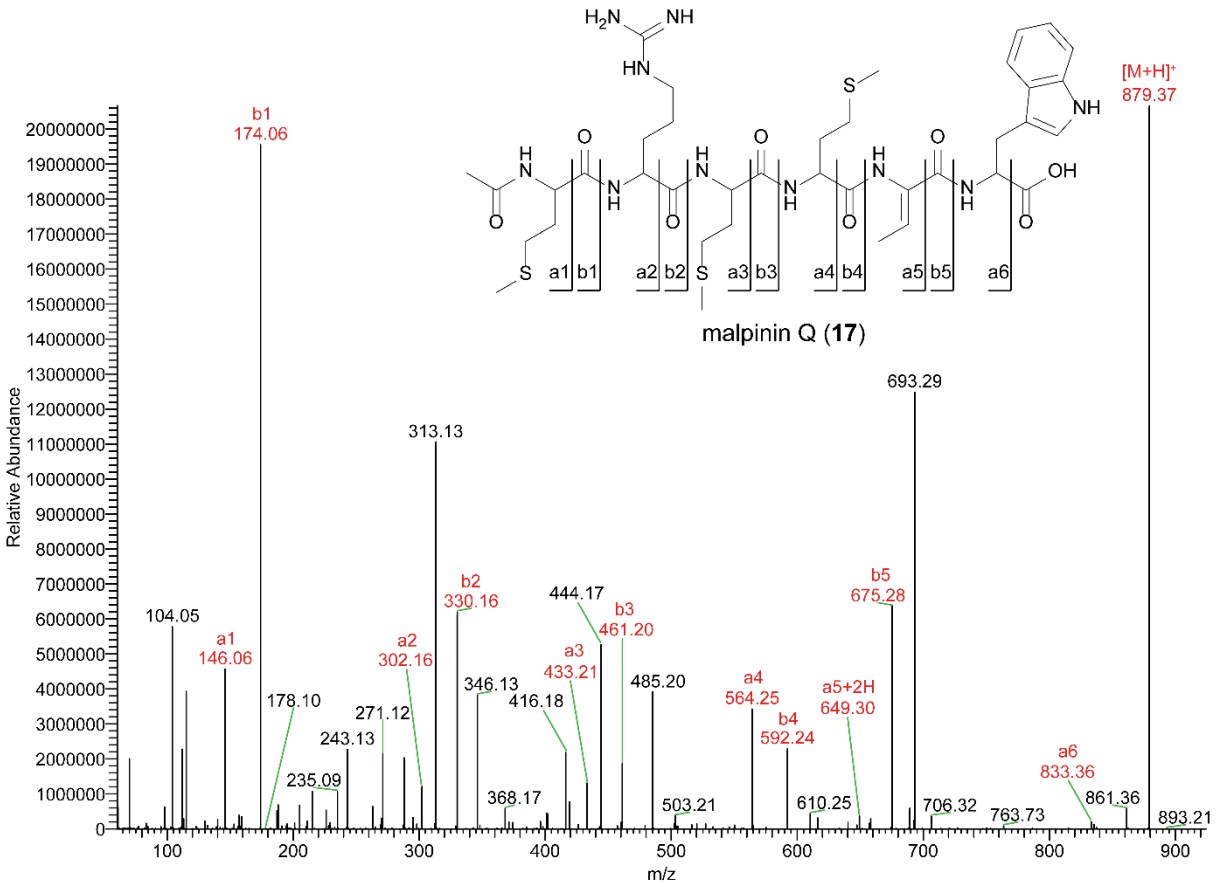


Figure S23. HR-ESI-MS/MS spectrum of 17.

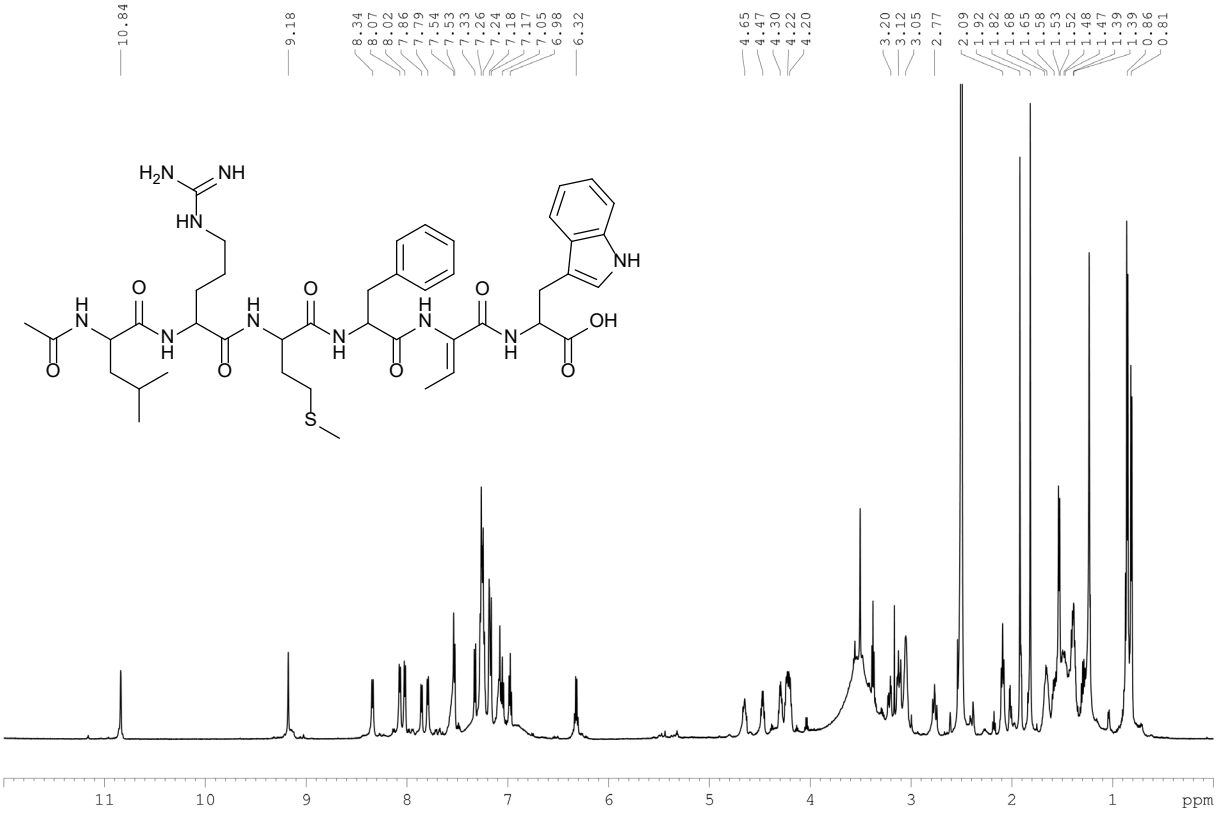


Figure S24. ^{1}H NMR spectrum of 6 in DMSO-d_6.

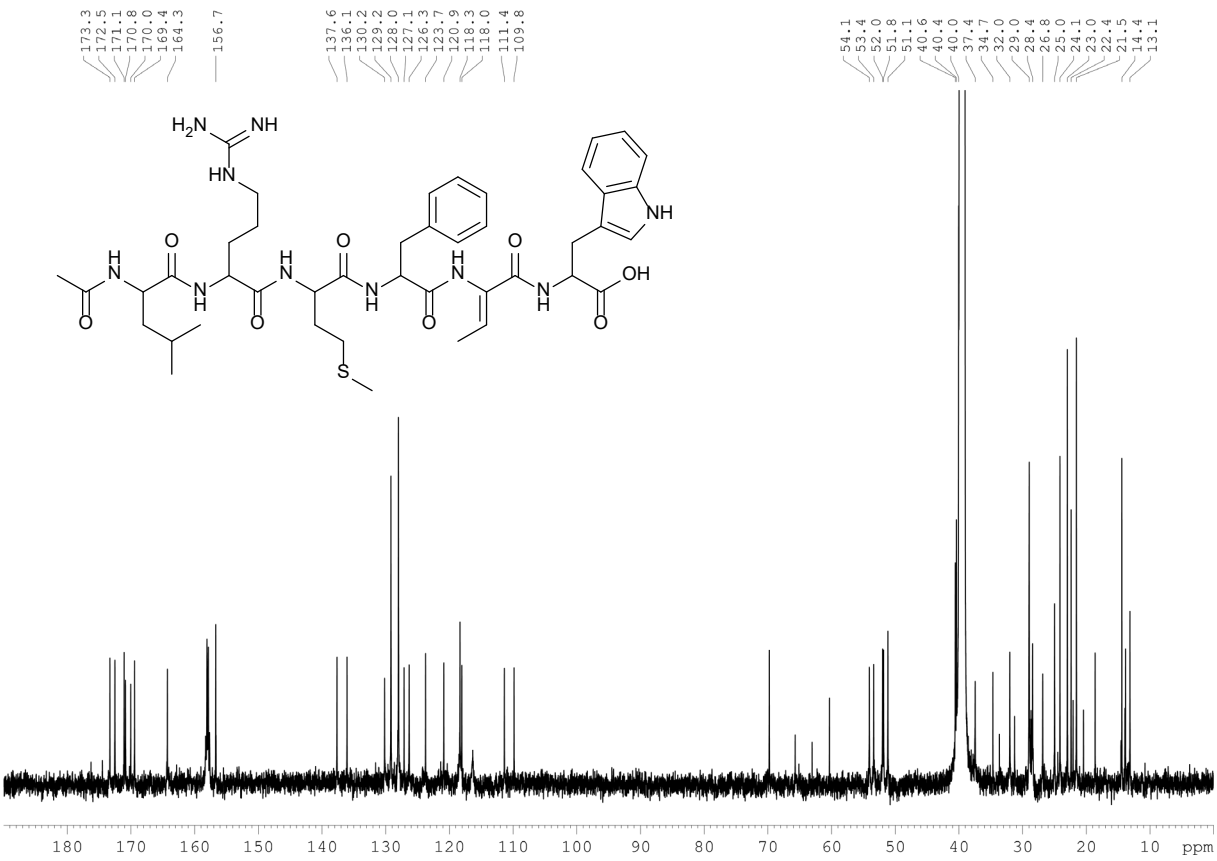


Figure S25. ^{13}C NMR spectrum of 6 in DMSO-d_6.

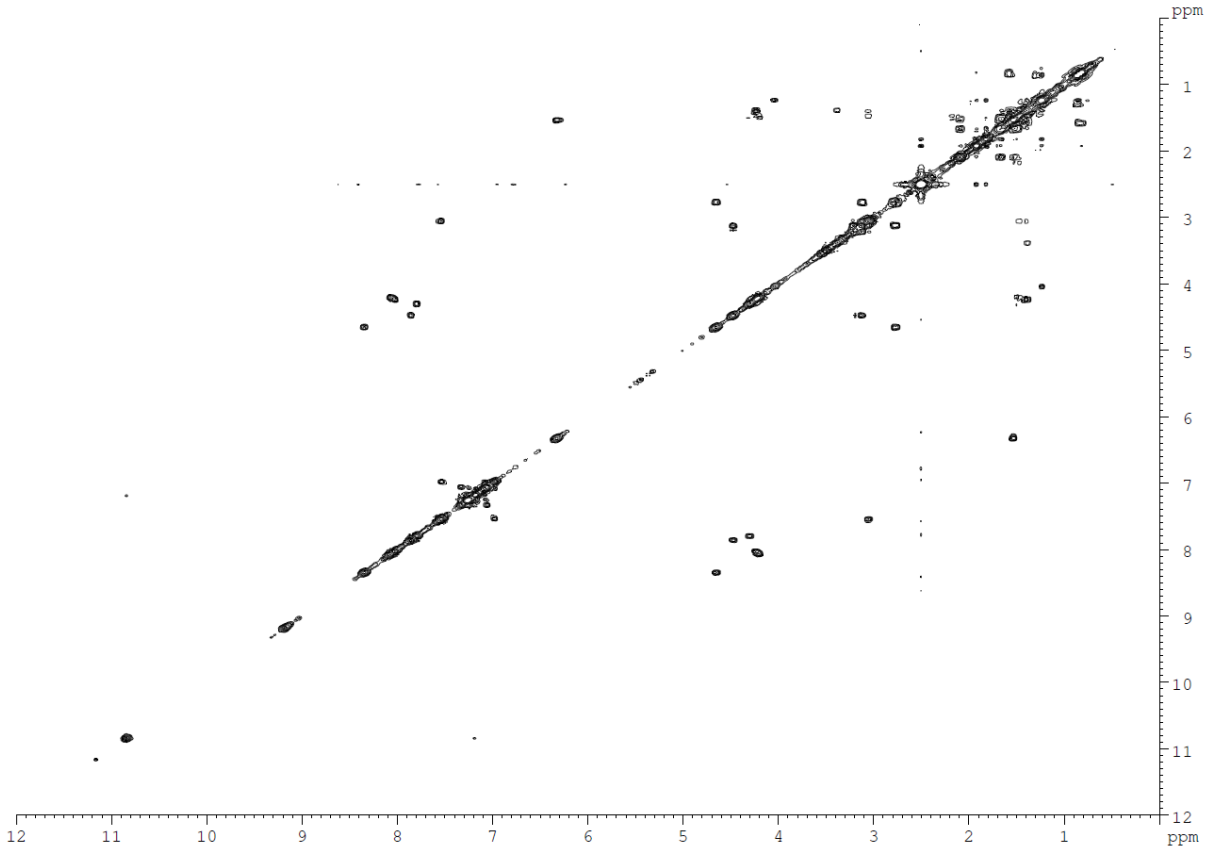


Figure S26. ^{1}H,^{1}H COSY NMR spectrum of 6 in DMSO-d_6.

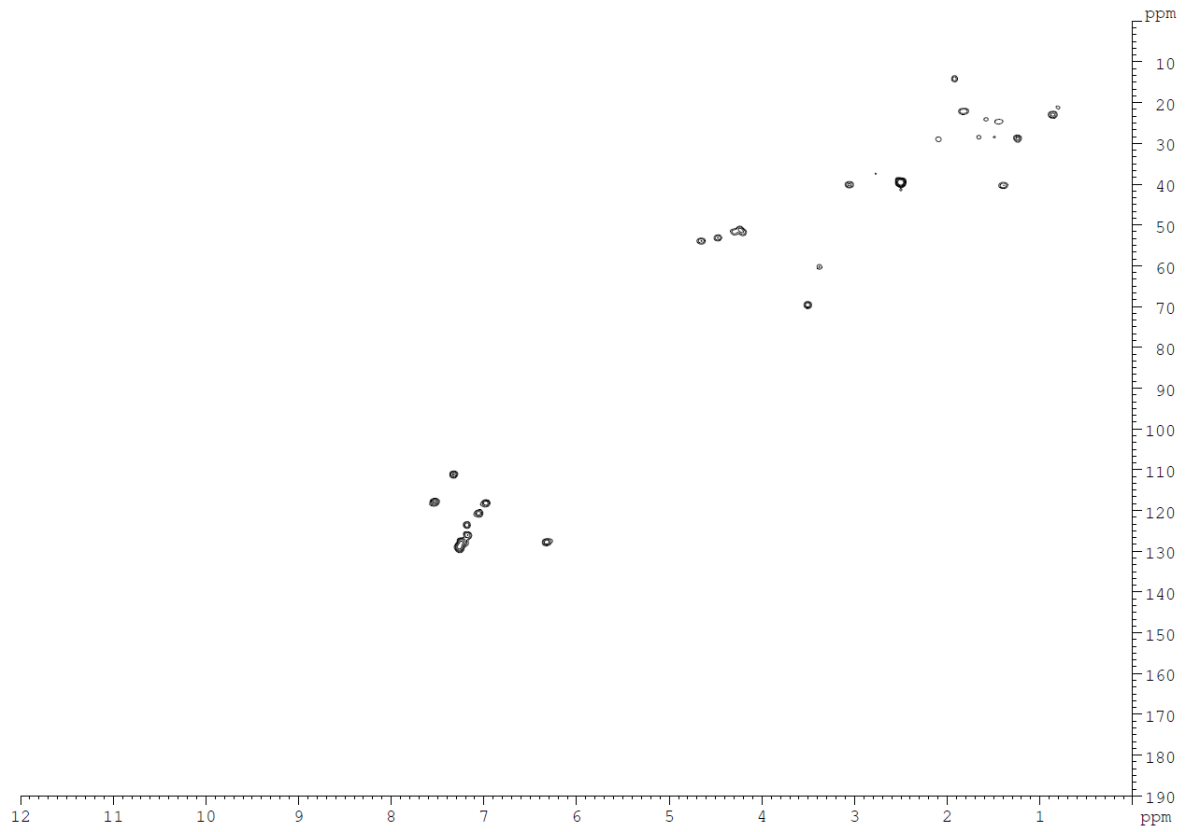


Figure S27. $^{1}H,^{13}C$ HSQC NMR spectrum of 6 in DMSO-d_6.

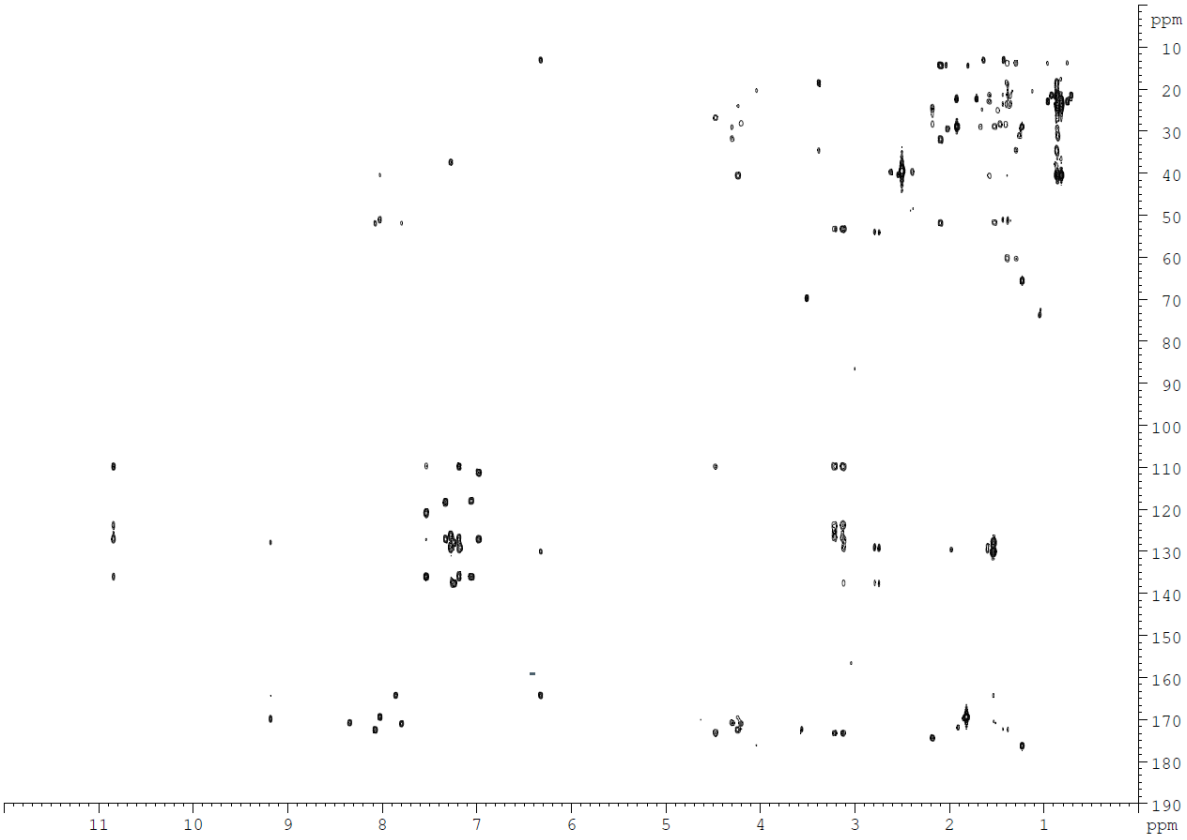


Figure S28. $^{1}H,^{13}C$ HMBC NMR spectrum of 6 in DMSO-d_6.

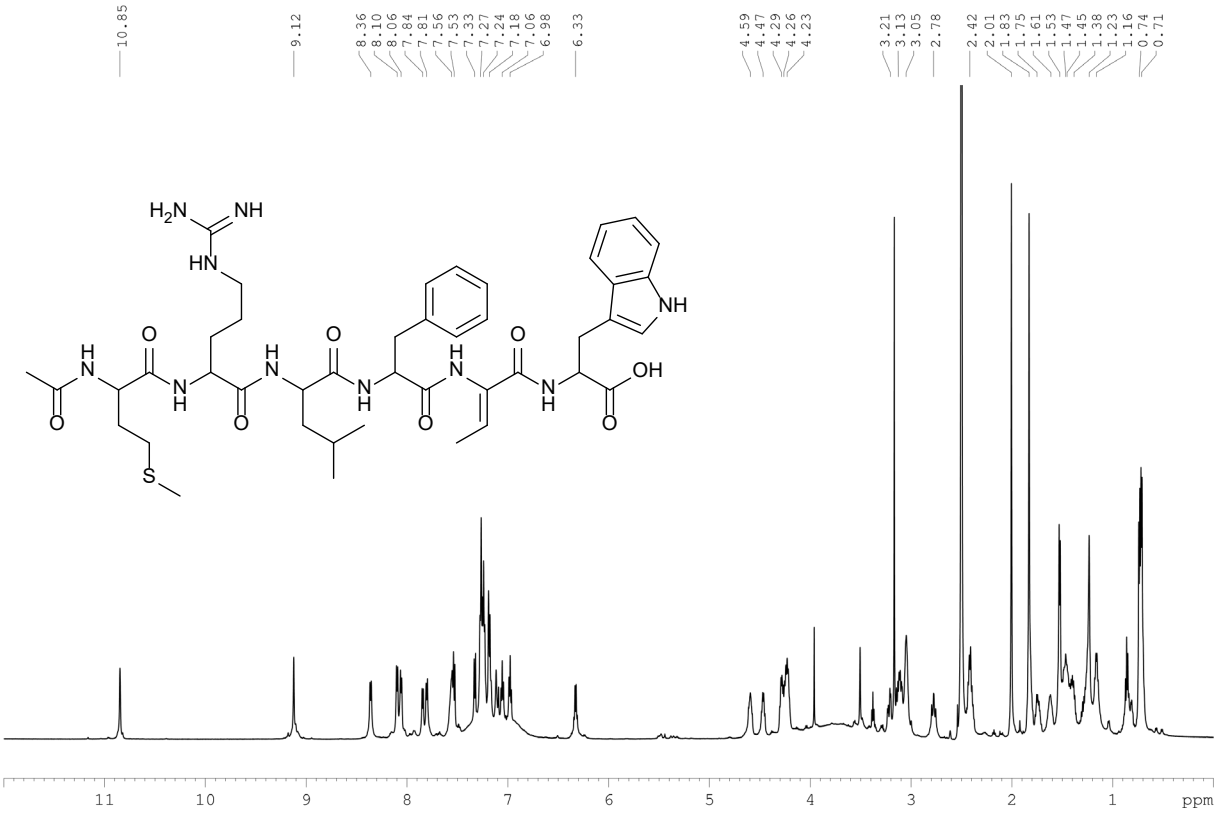


Figure S29. ^{1}H NMR spectrum of 7 in DMSO-d_6.

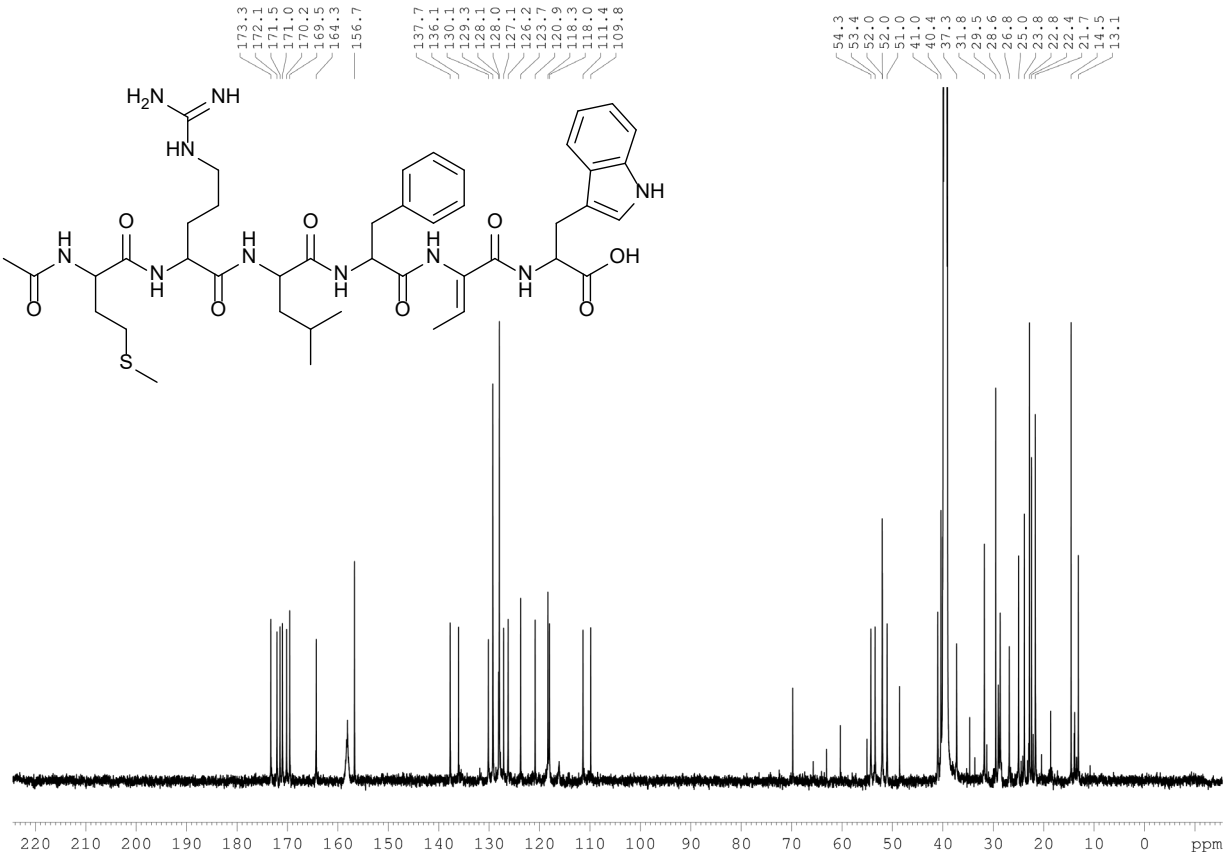


Figure S30. ^{13}C NMR spectrum of 7 in DMSO-d_6.

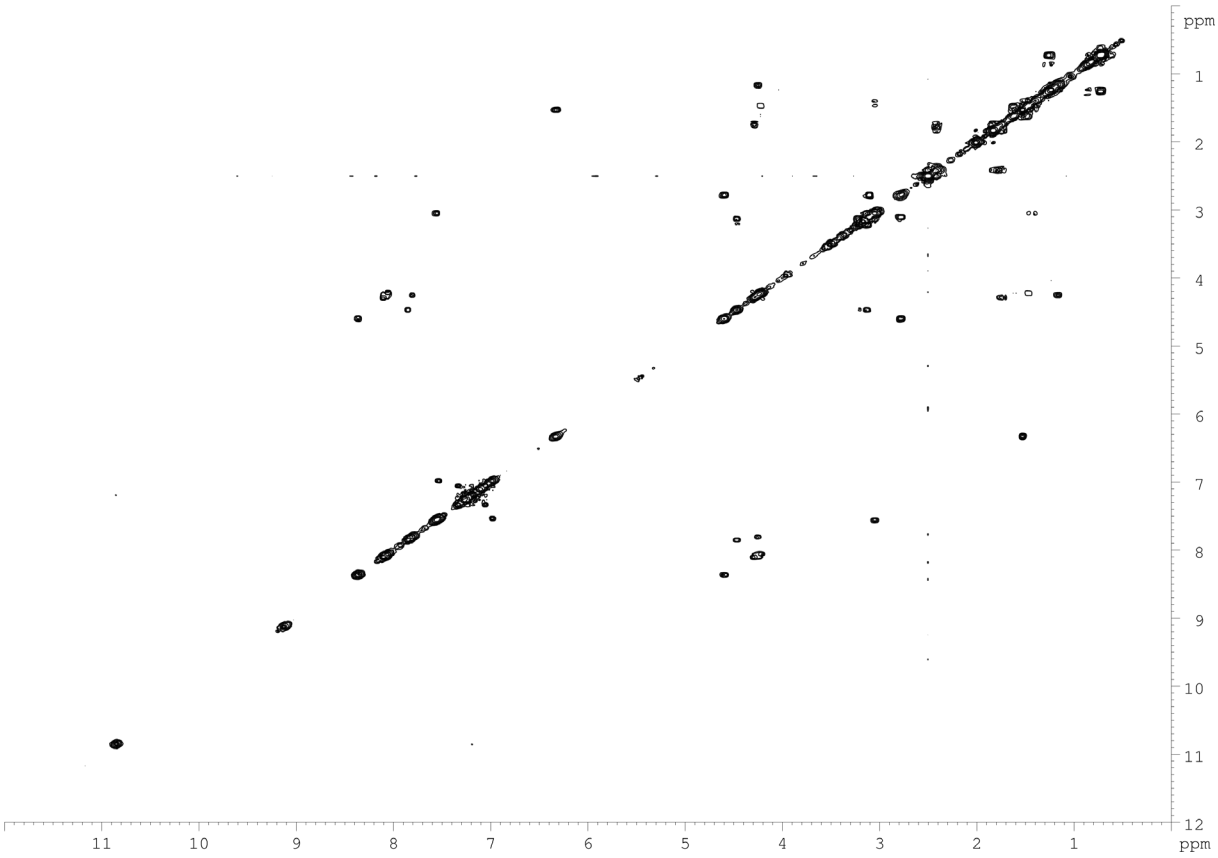


Figure S31. ^{1}H,^{1}H COSY NMR spectrum of 7 in DMSO-d_6.

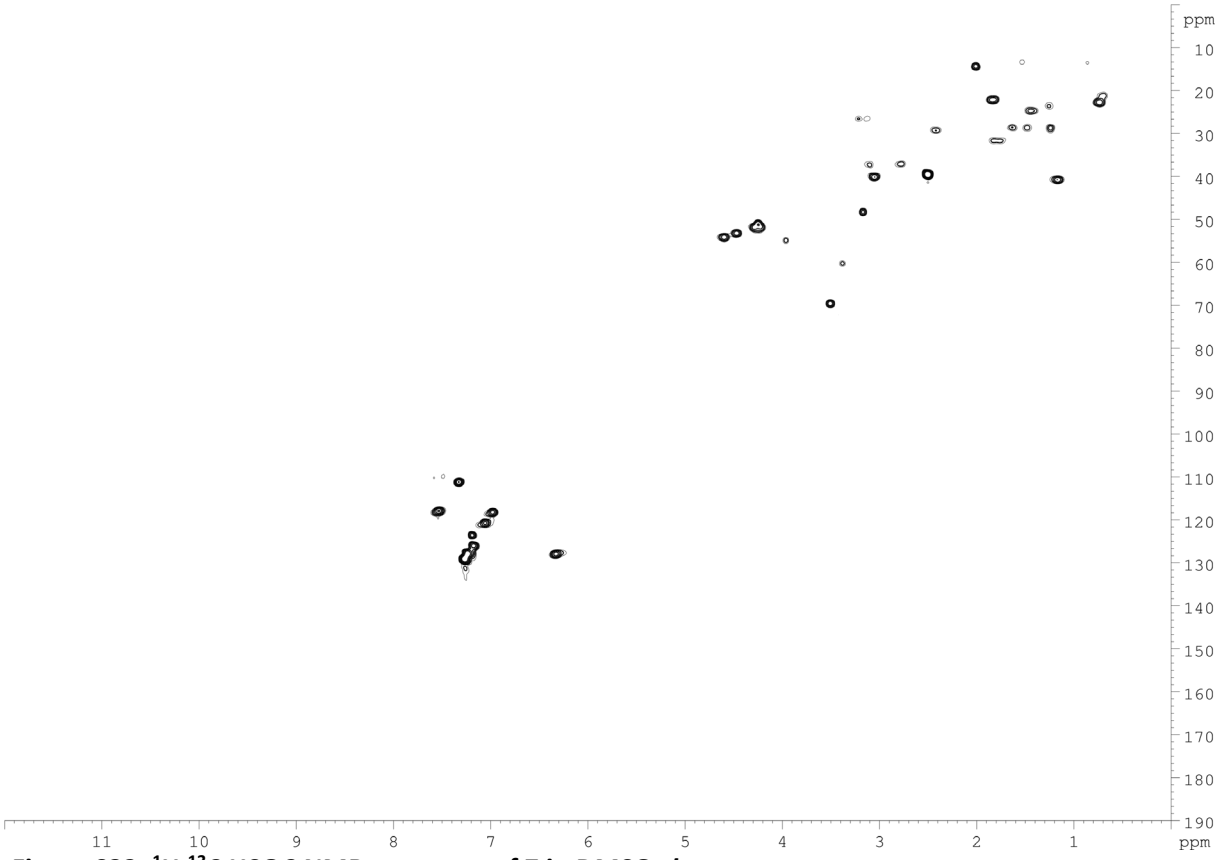


Figure S32. ^{1}H,^{13}C HSQC NMR spectrum of 7 in DMSO-d_6.

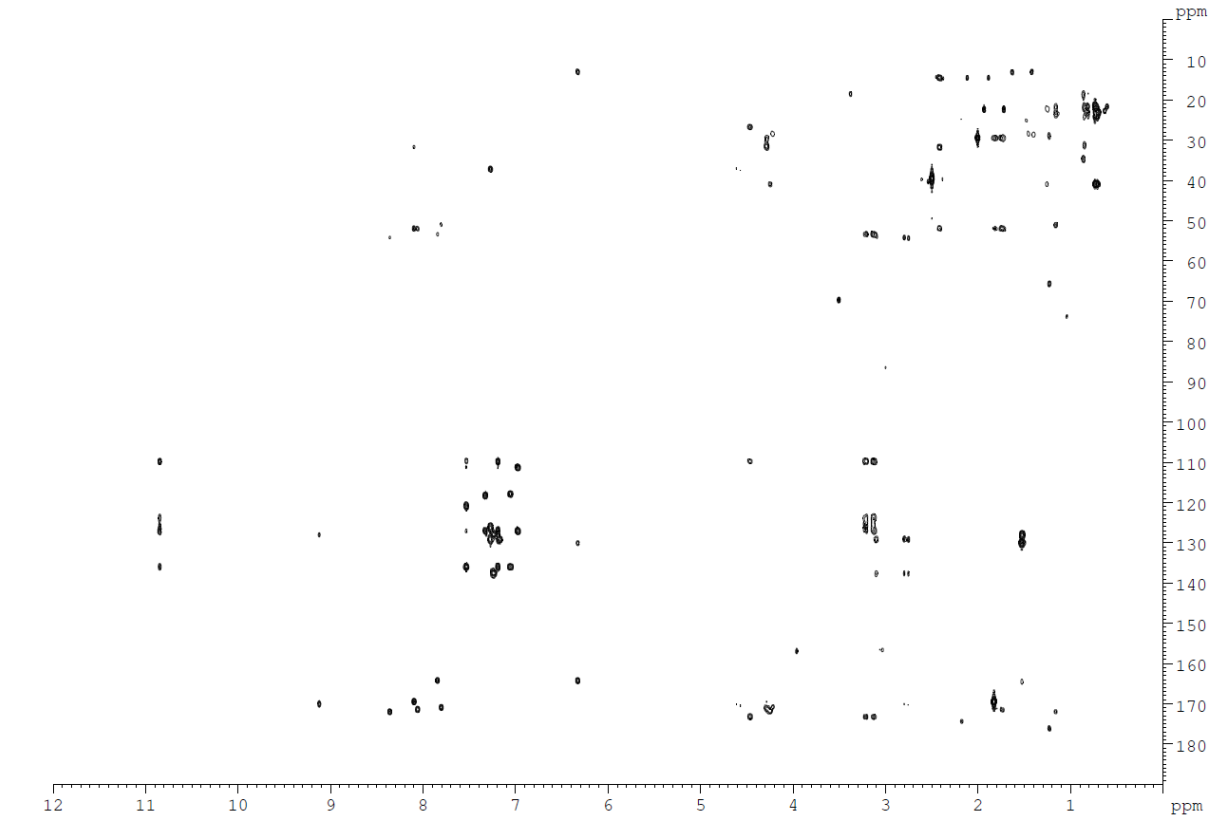


Figure S33. $^{1}H,^{13}C$ HMBC NMR spectrum of 7 in DMSO-d_6.

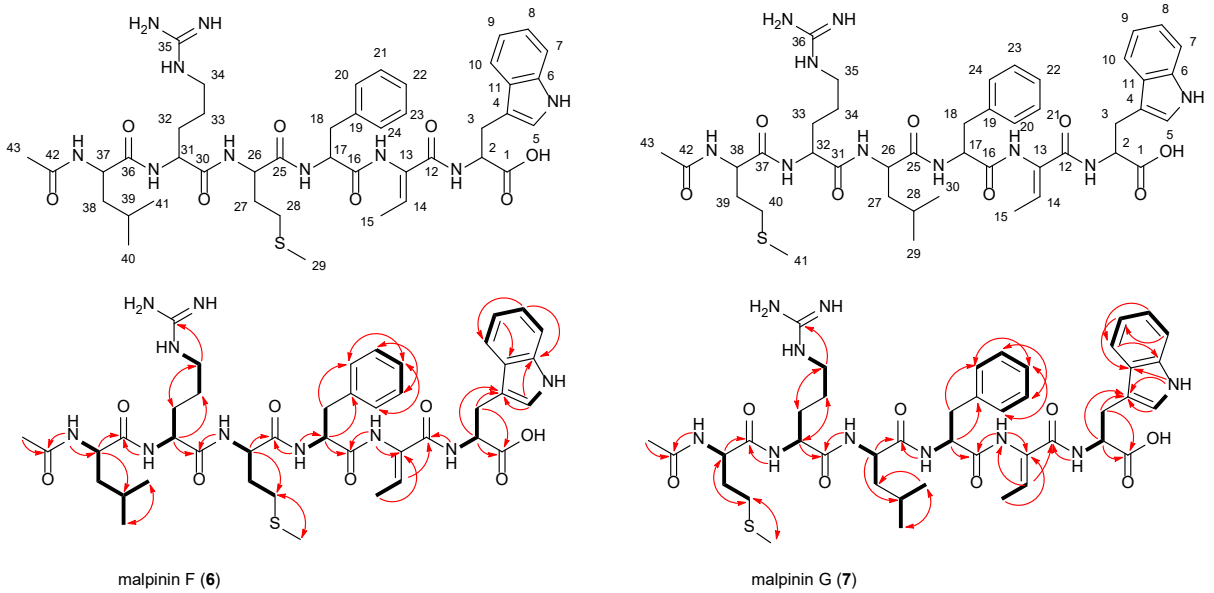


Figure S34. Carbon numbering, COSY and HMBC correlation of 6 and 7. COSY key correlations and HMBC key correlations are indicated as bold lines and red arrows, respectively.

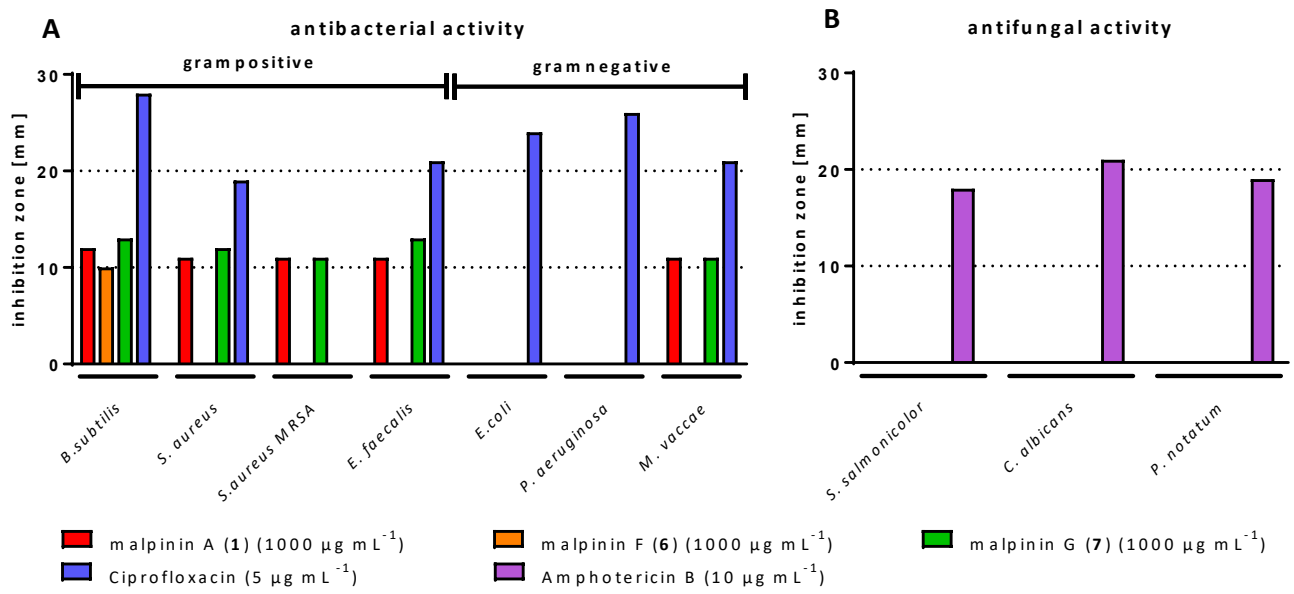


Figure S35. Antimicrobial activities of 1, 6 and 7. Metabolites were tested against bacteria (**A**) and fungi (**B**) using ciprofloxacin or amphotericin B as positive controls.

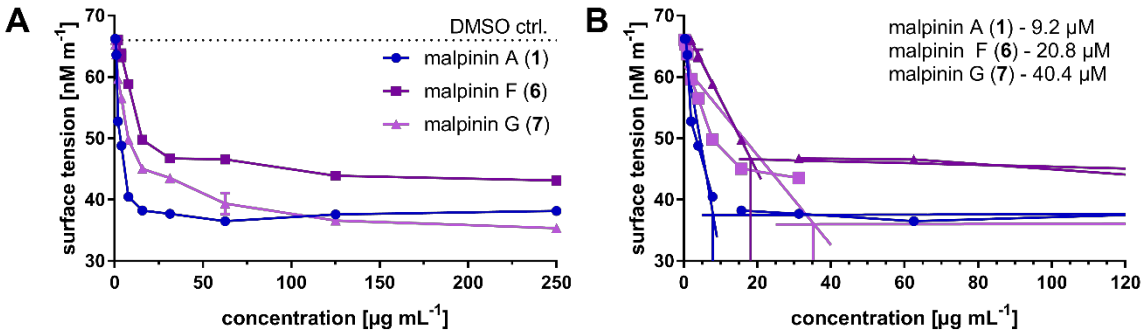


Figure S36. Surface tension lowering activity of 1, 6 and 7. Surface tension reducing activity of pure **1, 6** and **7** in comparison to 10% DMSO which was used as solvent. Error bars represents the standard error of the mean (SEM). **B**. Determination of the critical micelle concentration (CMC) by point of intersection of linear regression of plateau and lowering range.

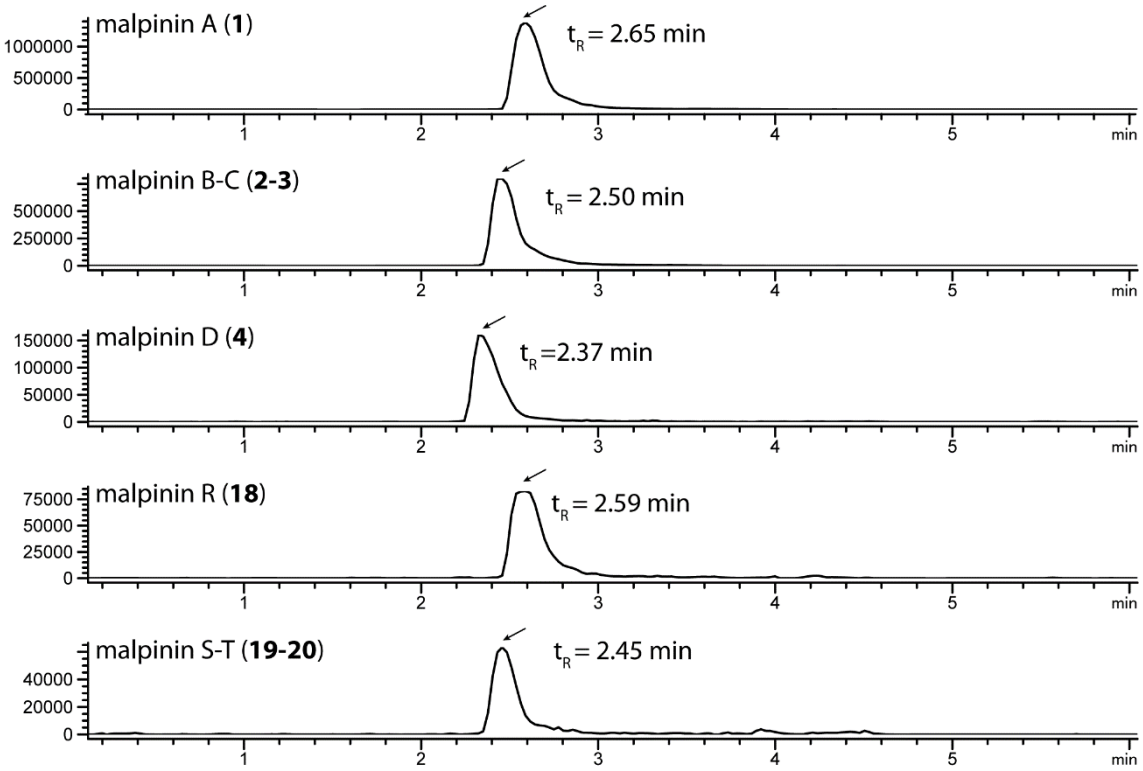


Figure S37. Extracted ion chromatograms (EICs) of metabolite extracts from cultures supplemented with L-tryptophan, recorded by UHPLC method B (Table S16).

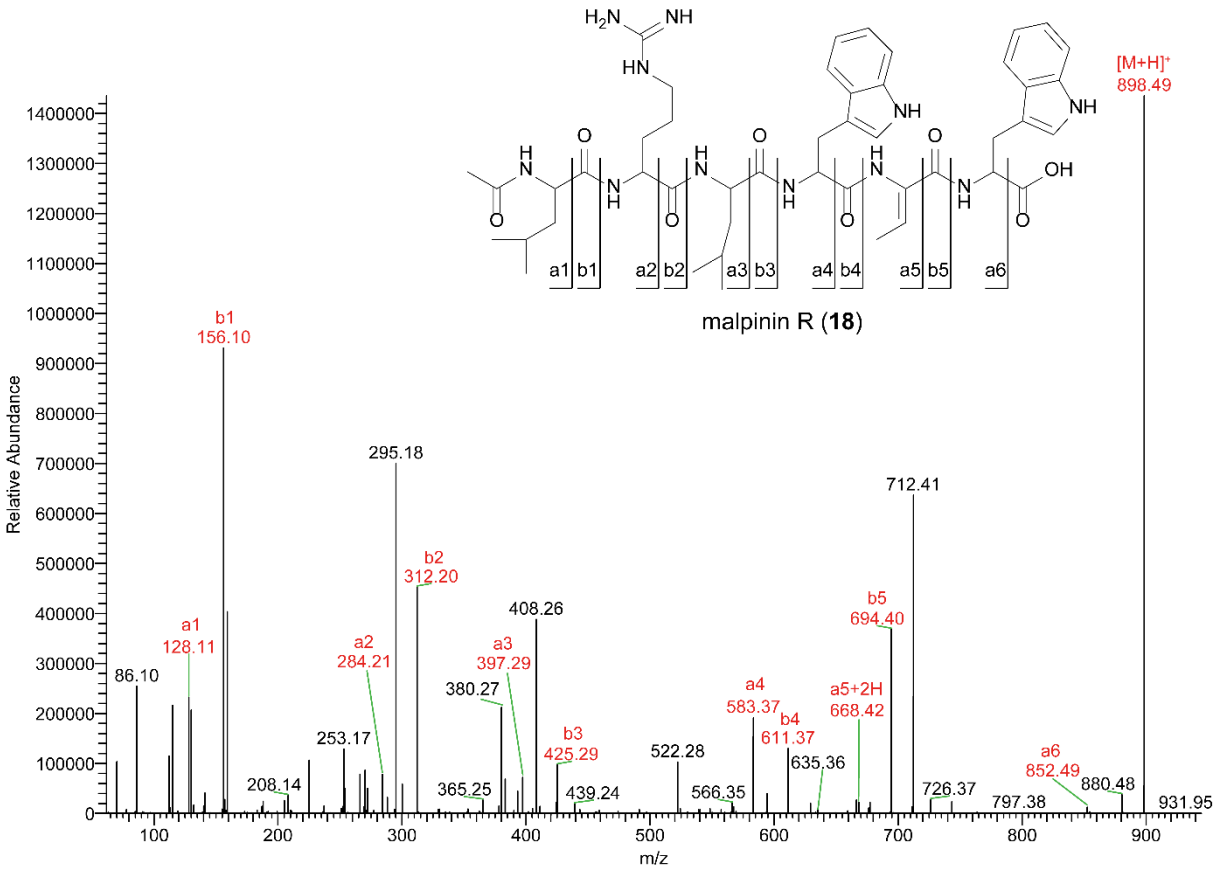


Figure S38. HR-ESI-MS/MS spectrum of 18.

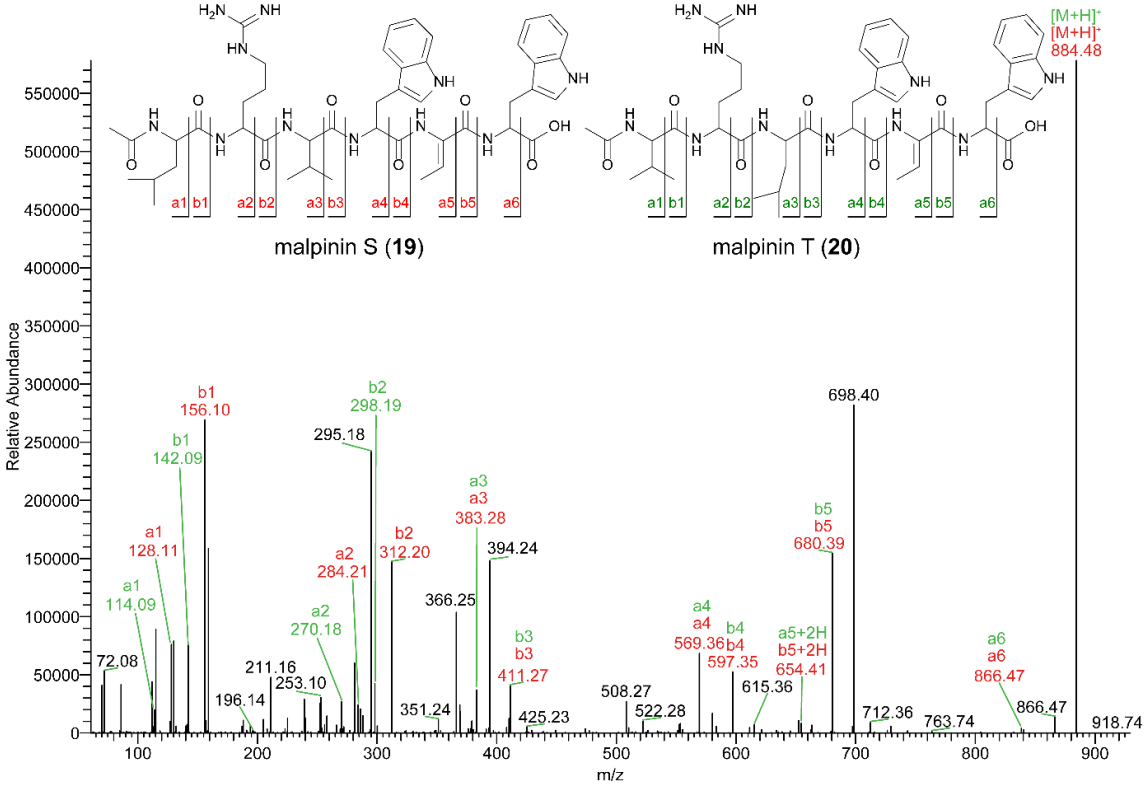


Figure S39. HR-ESI-MS/MS spectrum of the two coeluting isomers 19 and 20.

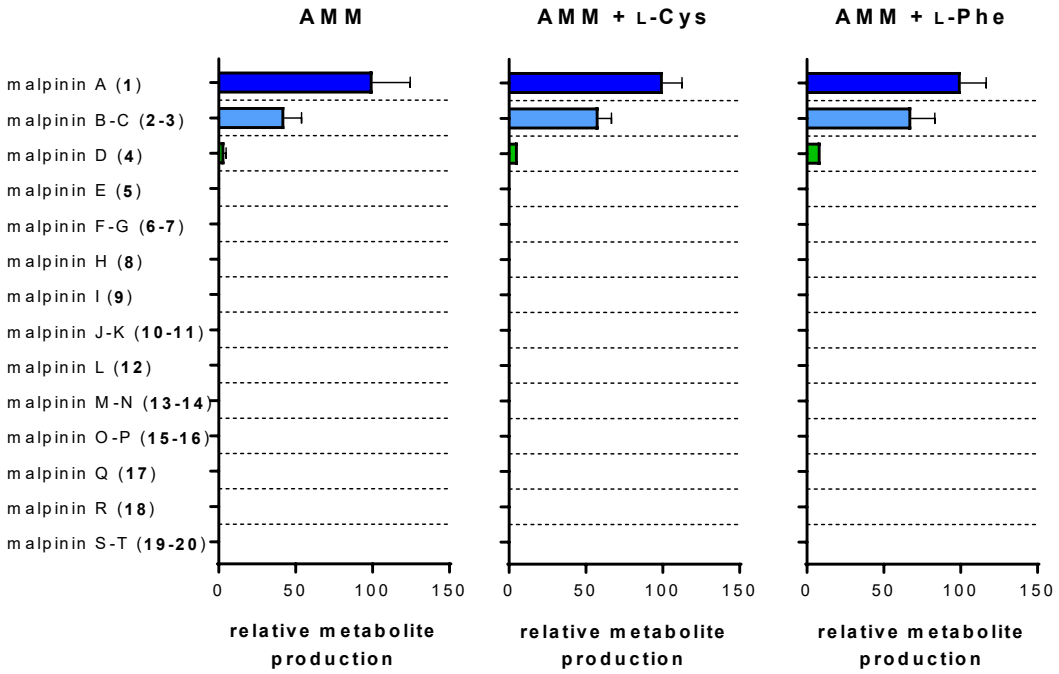


Figure S40. Quantification of malpinins in *M. alpina* cultures by UHPLC-MS. AMM medium was supplemented with 5 mM amino acids. Note that the metabolic profile did not change regardless of the supplementation.

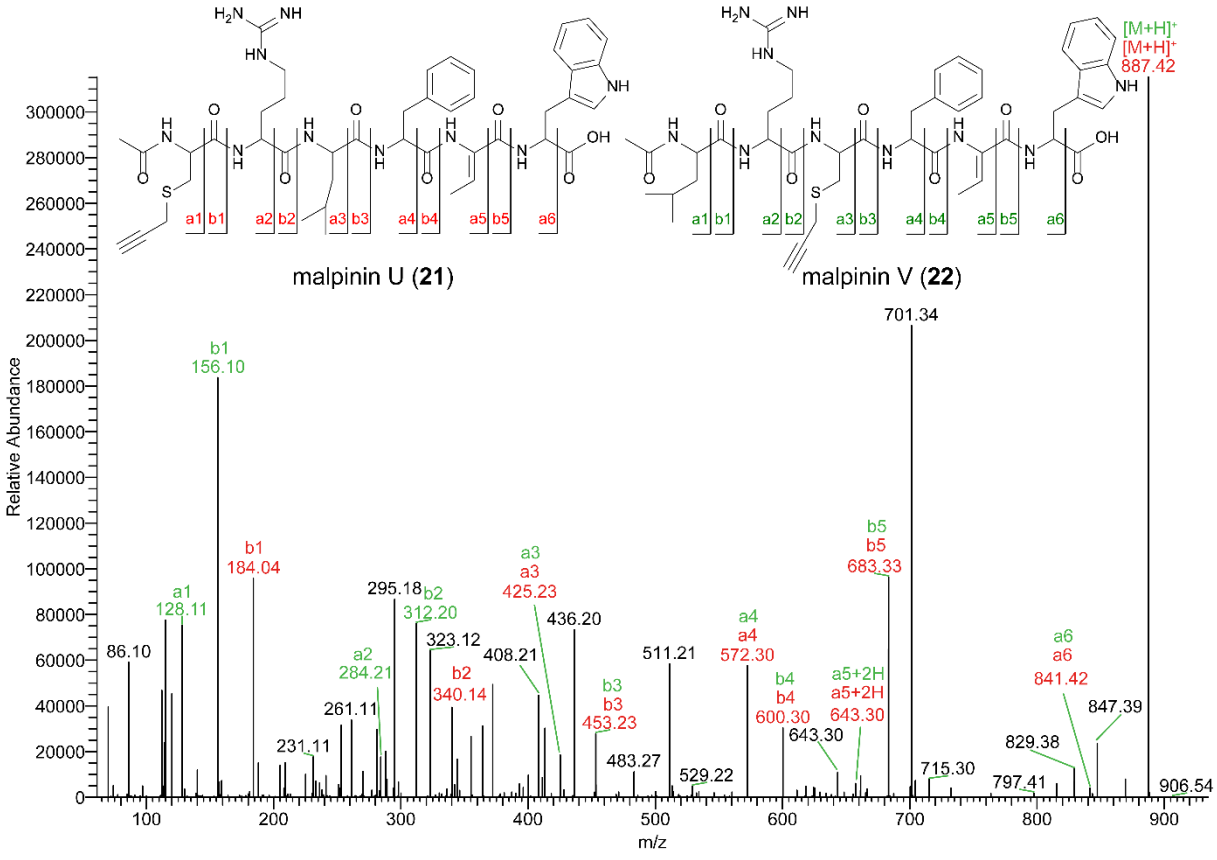


Figure S41. HR-ESI-MS/MS spectrum of the two coeluting isomers 21 and 22.

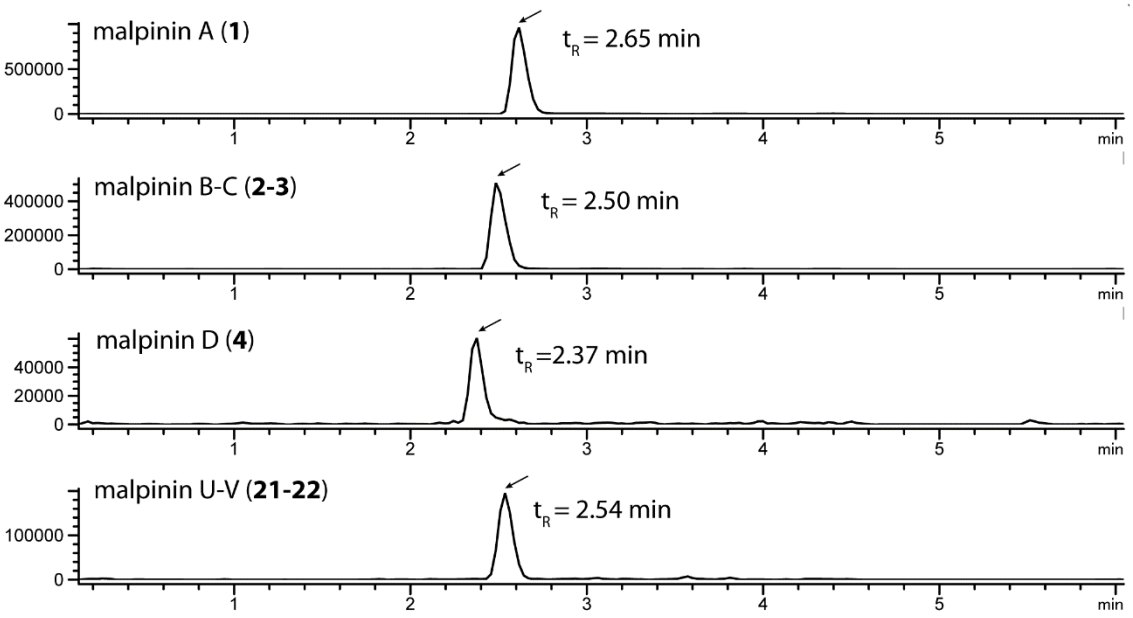


Figure S42. Extracted ion chromatograms (EICs) of metabolite extracts from cultures supplemented with S-propargyl-L-cysteine, recorded by UHPLC method B (Table S16).

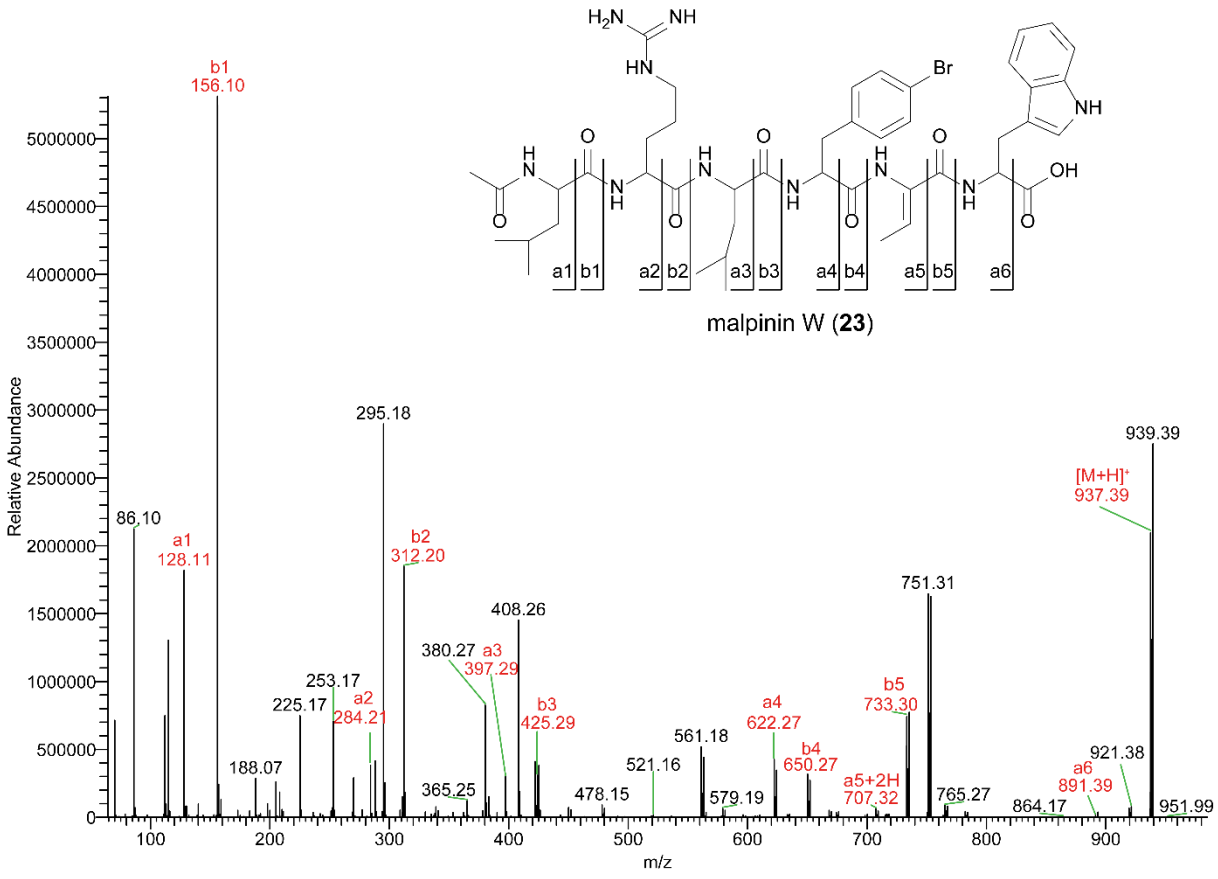


Figure S43. HR-ESI-MS/MS spectrum of 23.

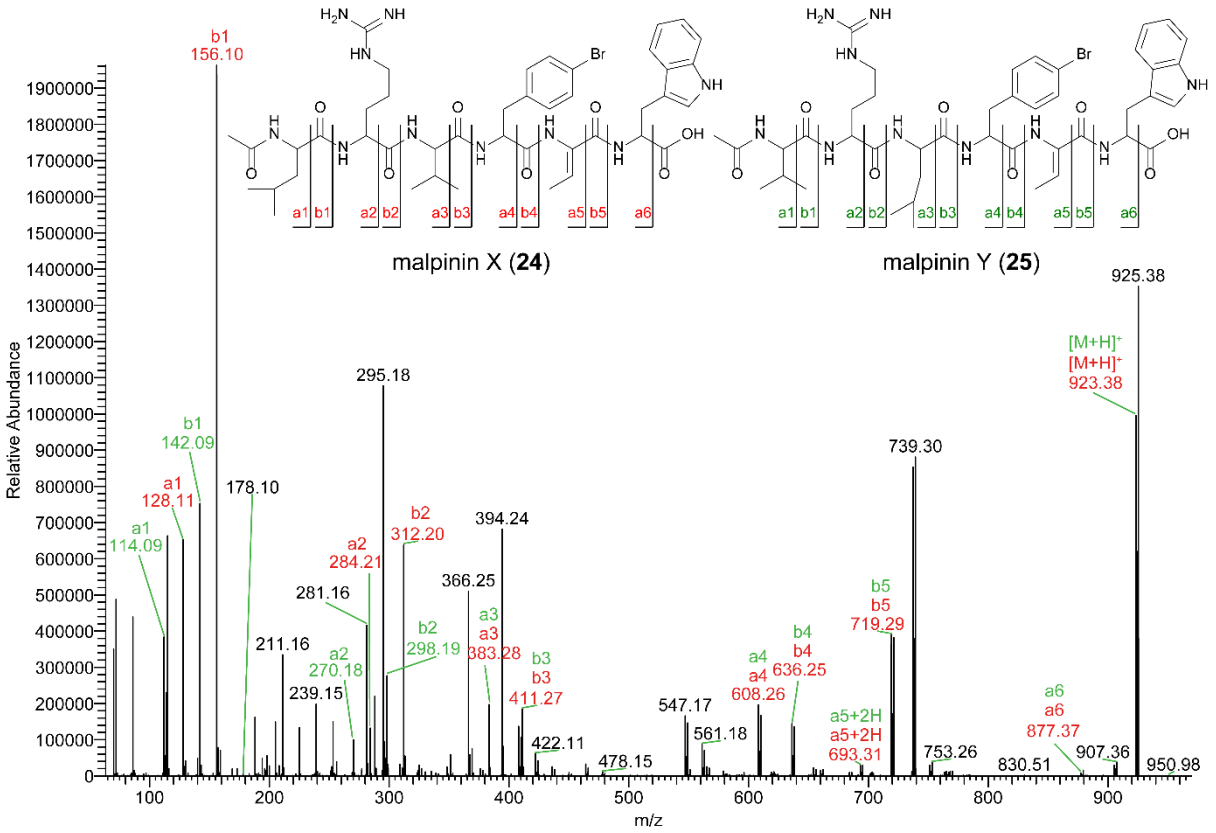


Figure S44. HR-ESI-MS/MS spectrum of the two coeluting isomers 24 and 25.

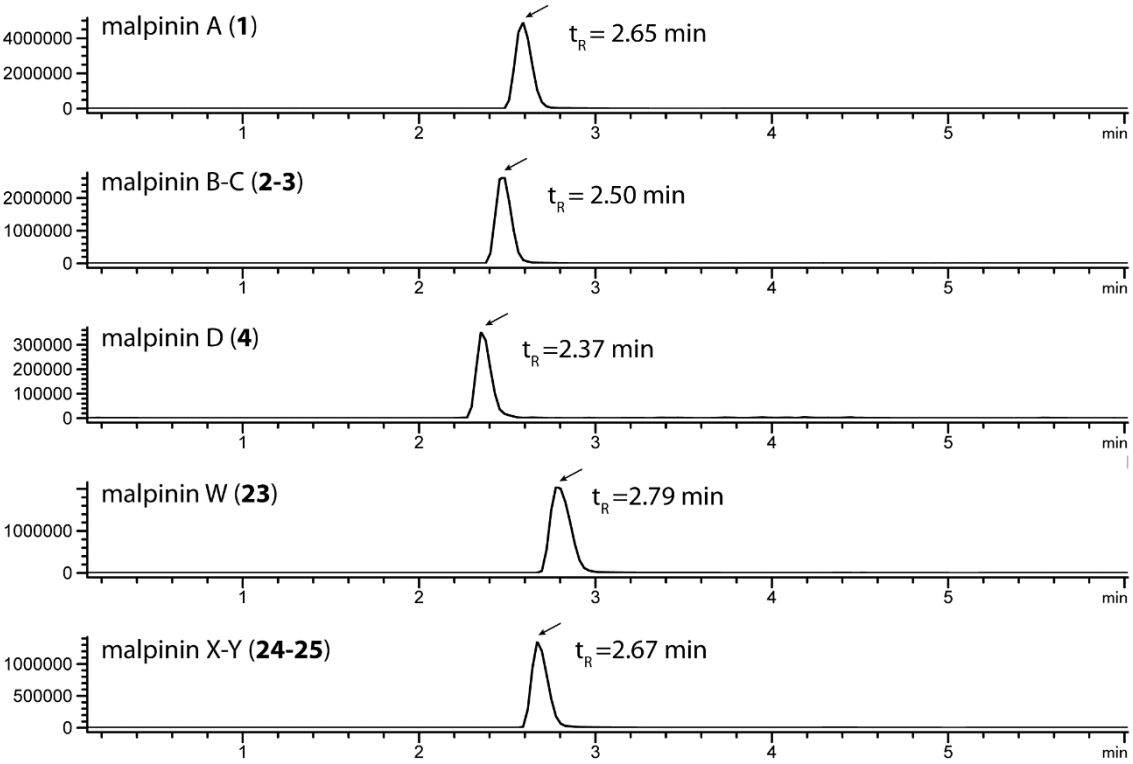


Figure S45. Extracted ion chromatograms (EICs) of metabolite extracts from cultures supplemented with 4-bromo-L-phenylalanine, recorded by UHPLC method B (Table S16).

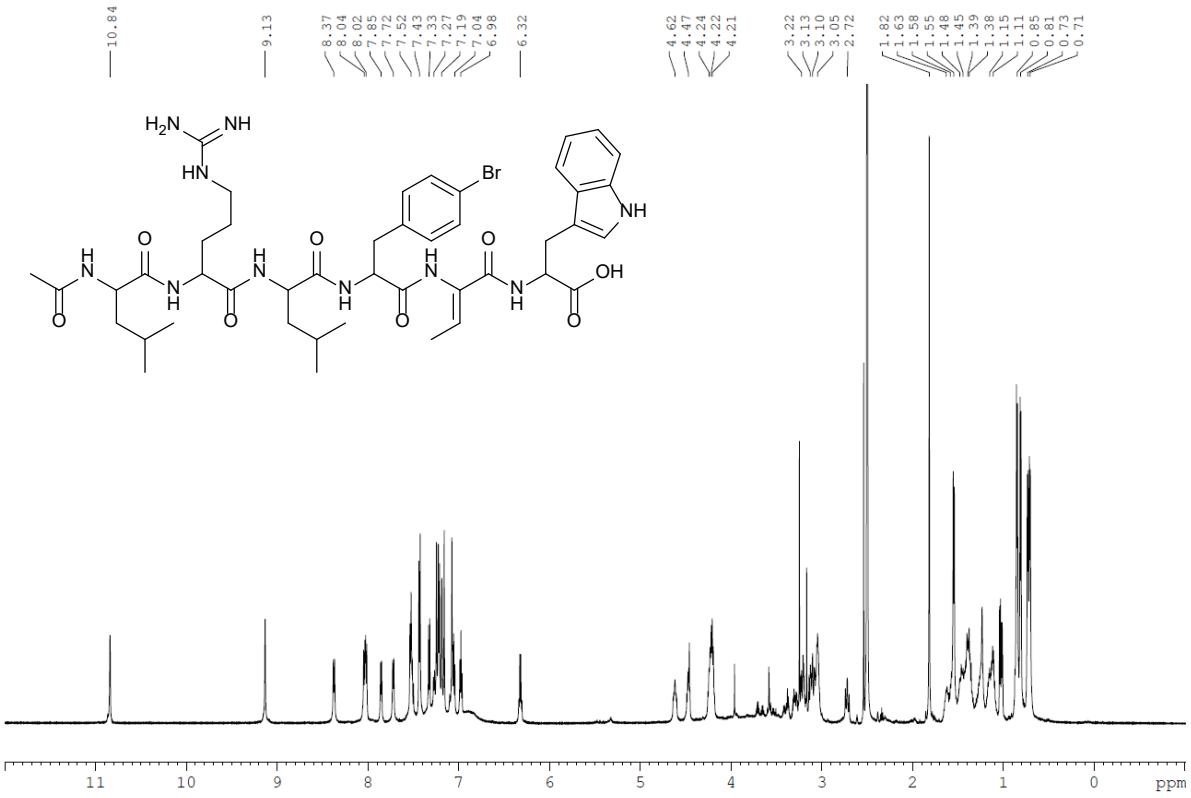


Figure S46. ^{1}H NMR spectrum of 23 in DMSO-d_6.

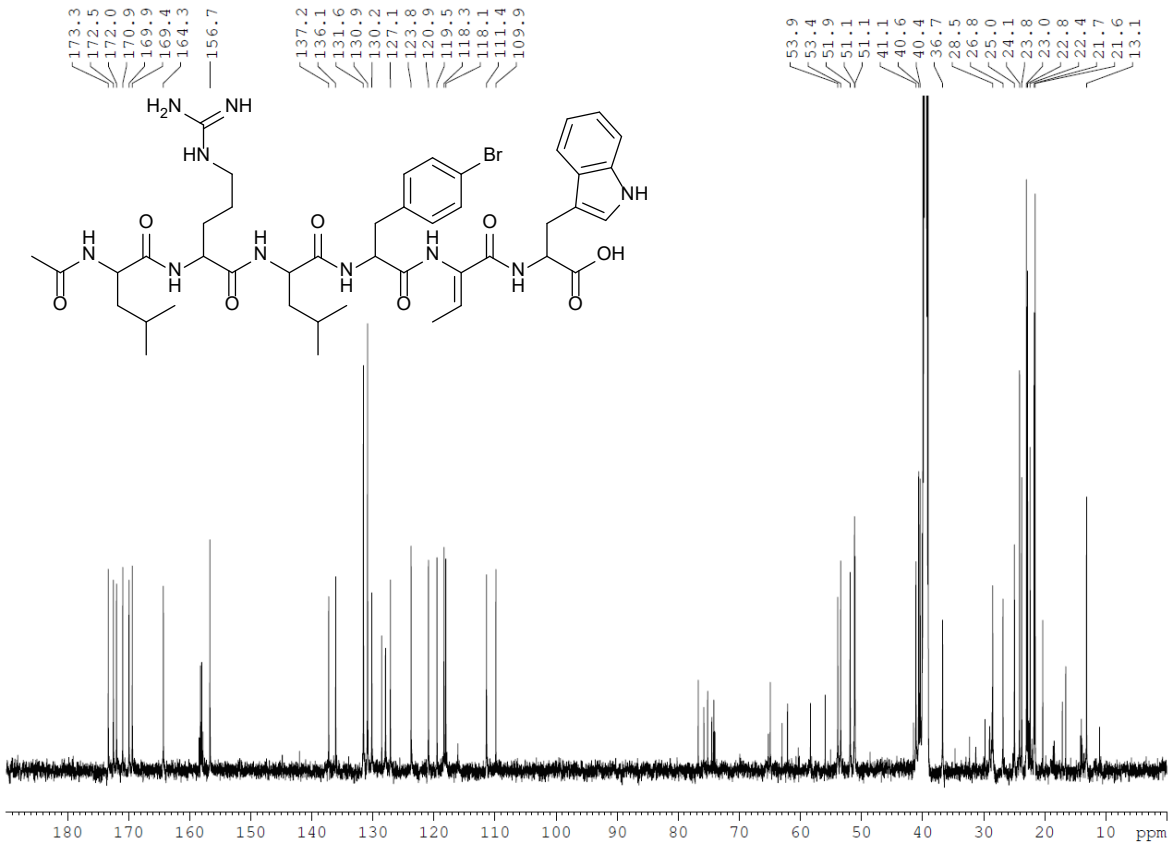


Figure S47. ^{13}C NMR spectrum of 23 in DMSO-d_6.

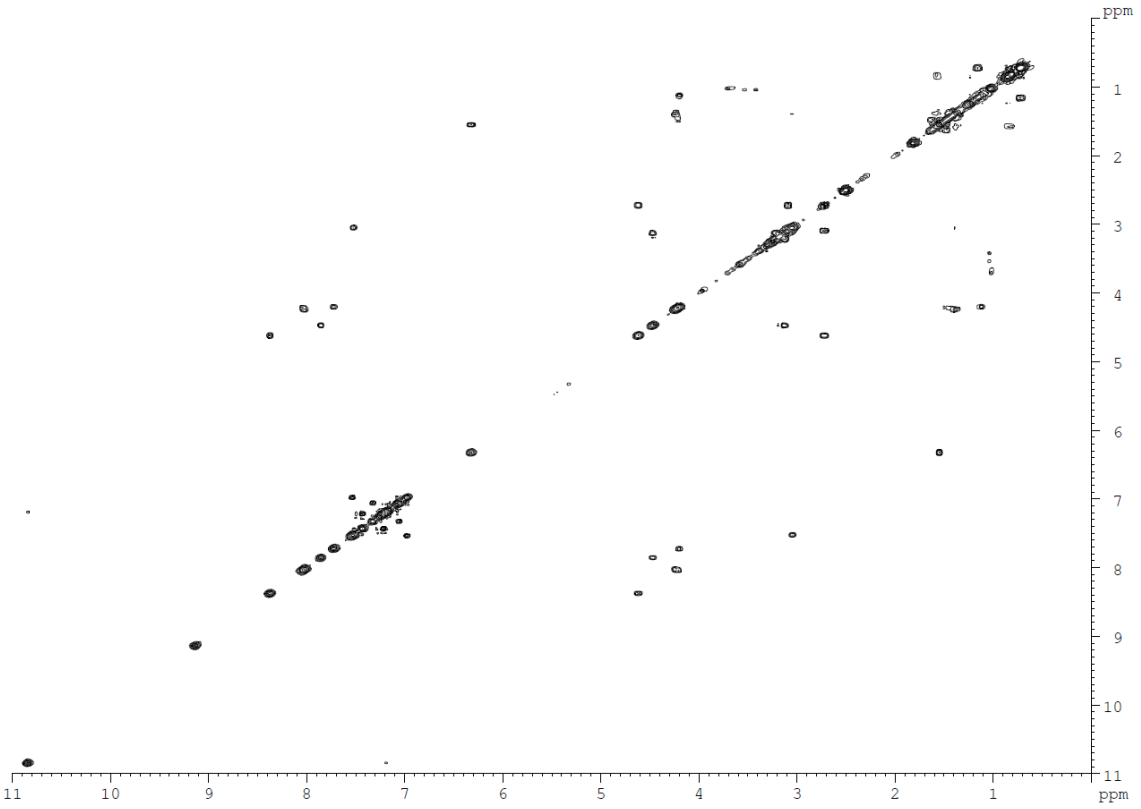


Figure S48. ^{1}H,^{1}H COSY NMR spectrum of 23 in DMSO-d_6.

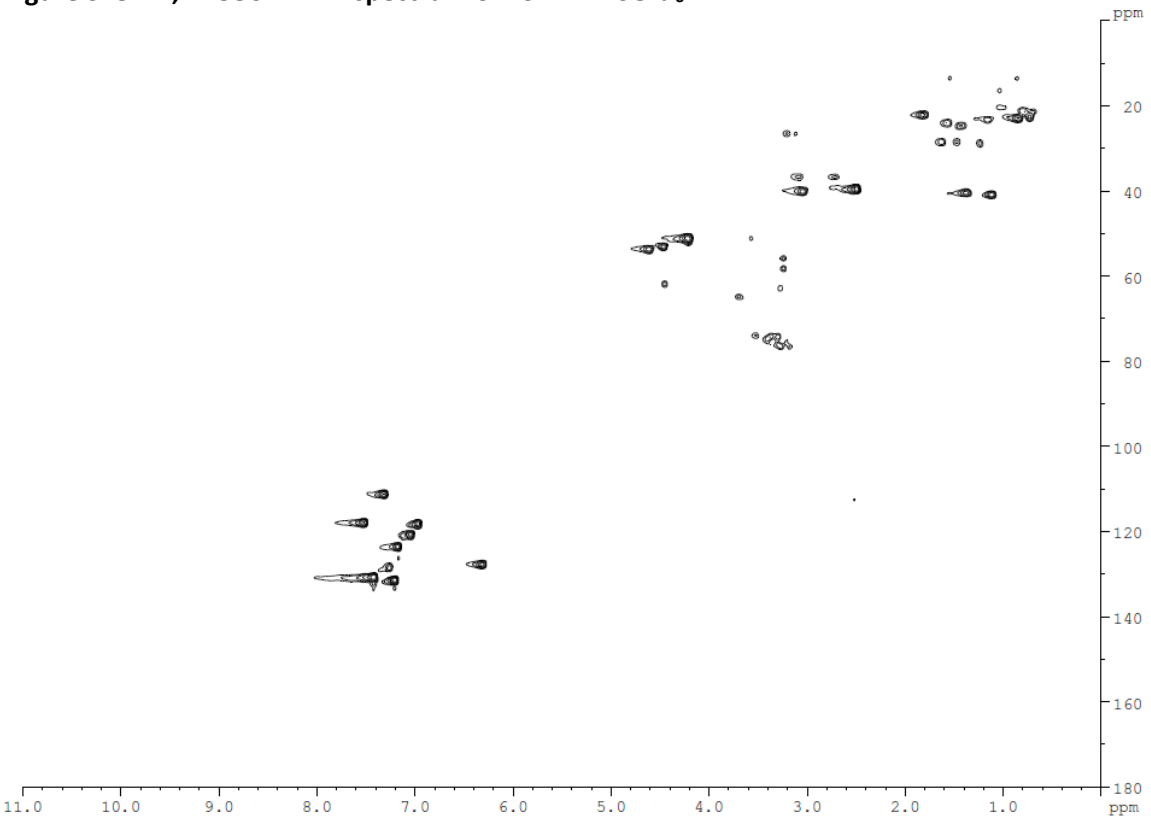


Figure S49. ^{1}H,^{13}C HSQC NMR spectrum of 23 in DMSO-d_6.

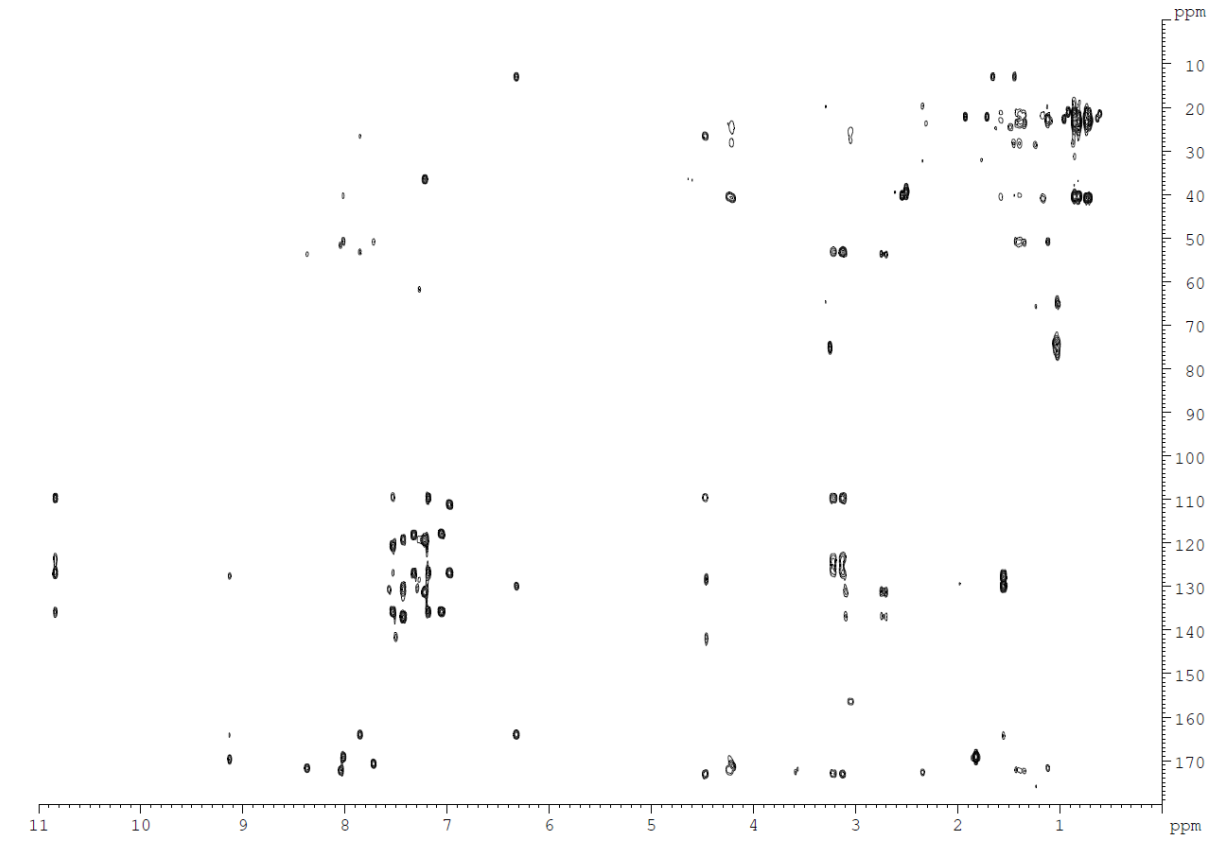


Figure S50. $^{1}H,^{13}C$ HMBC NMR spectrum of 23 in DMSO-d_6.

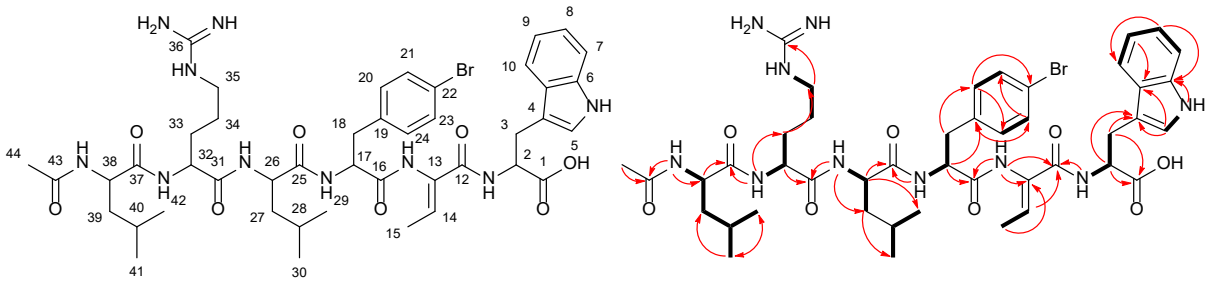


Figure S51. Carbon numbering, COSY and HMBC correlation of malpinin W (23). COSY key correlations and HMBC key correlations are indicated as bold lines and red arrows, respectively.

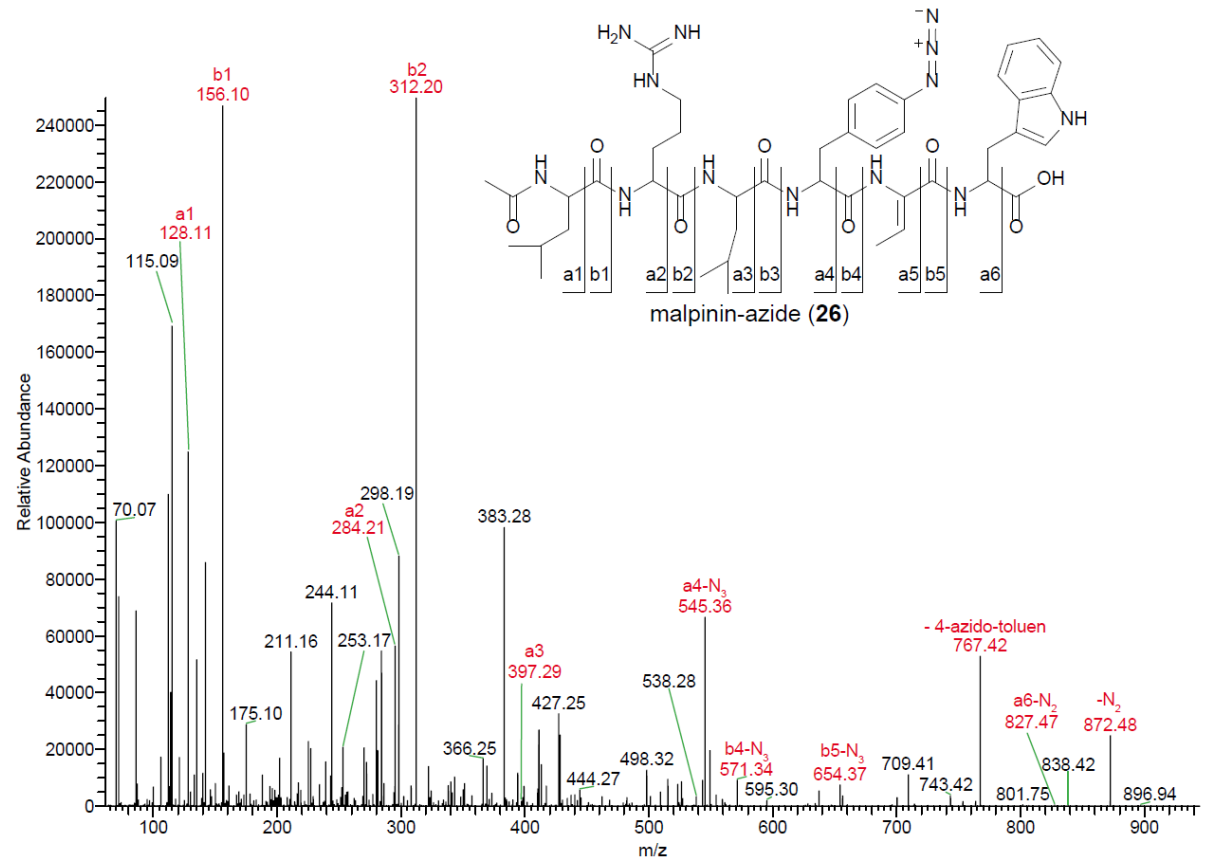


Figure S52. HR-ESI-MS/MS spectrum of 26.

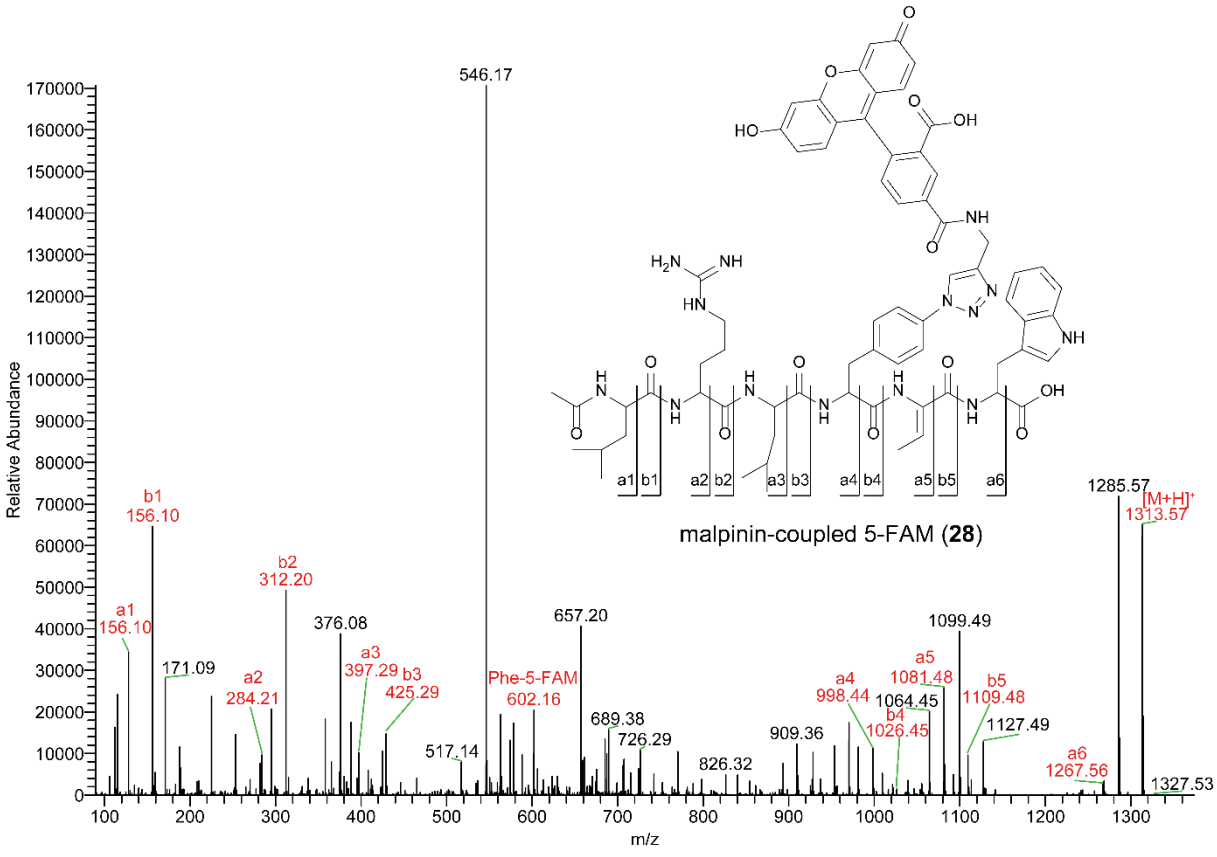


Figure S53. HR-ESI-MS/MS spectrum of 27.

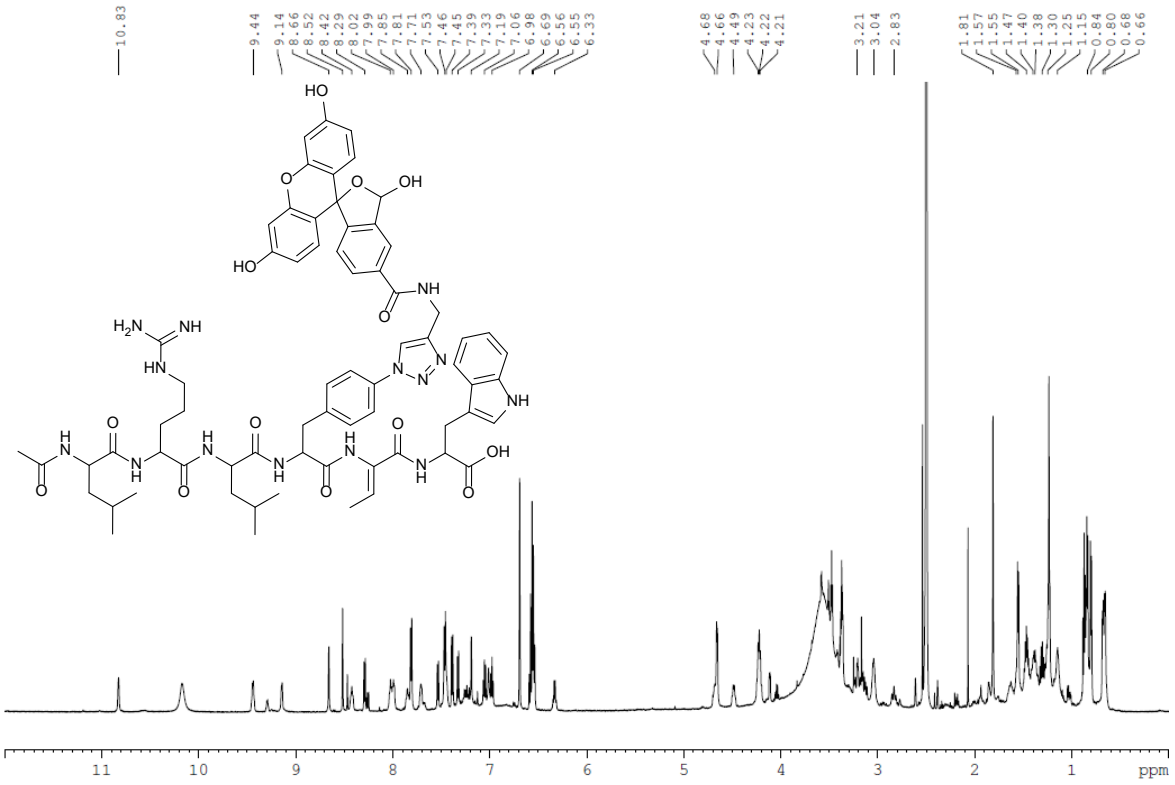


Figure S54. ^{1}H NMR spectrum of 27 in DMSO-d_6.

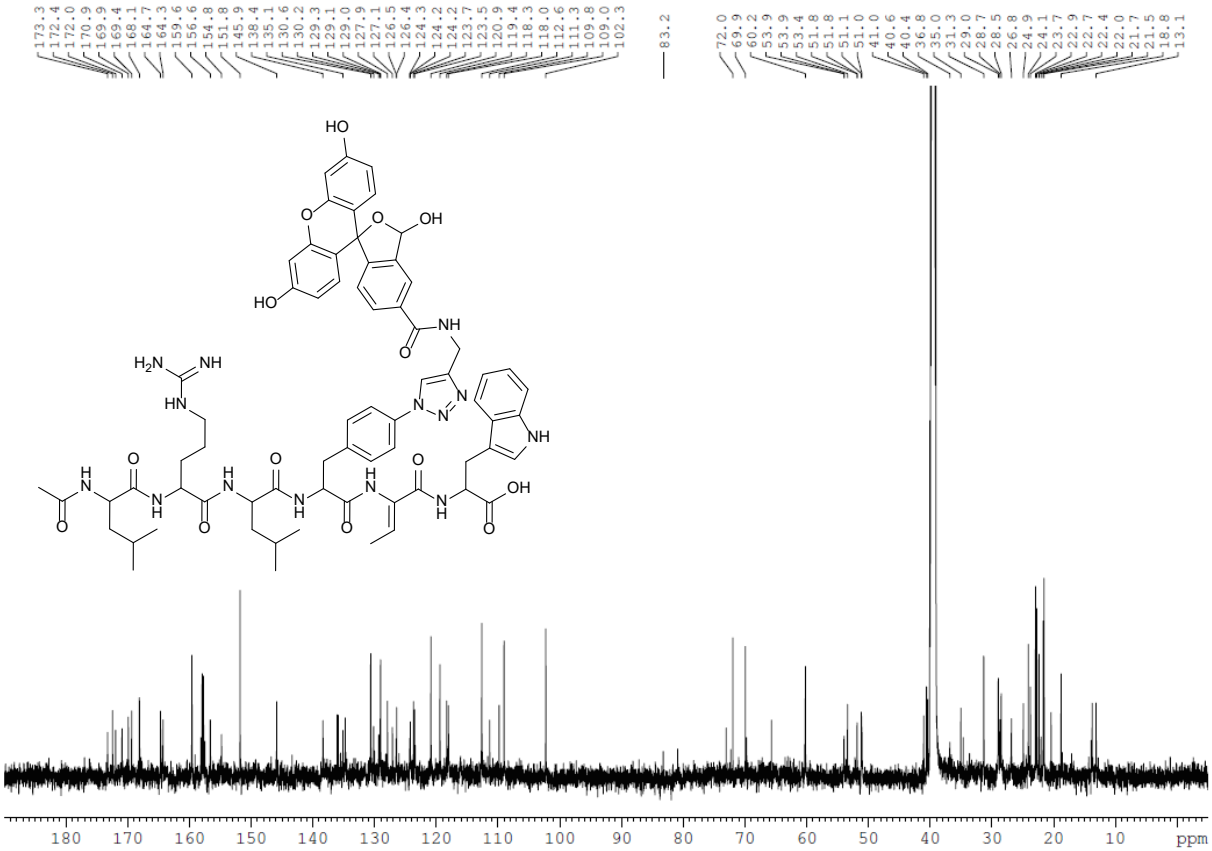


Figure S55. 13C NMR spectrum of 27 in DMSO-d_6.

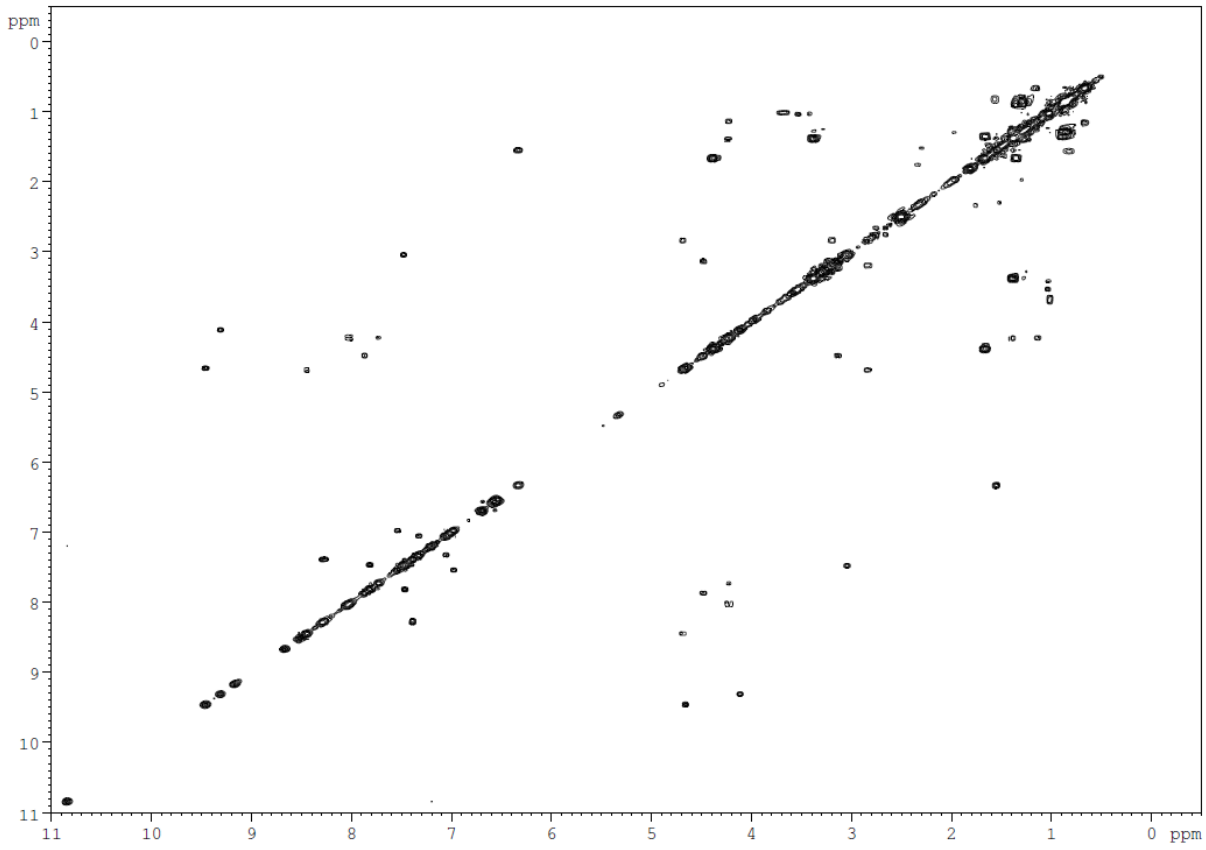


Figure S56. ^{1}H,^{1}H COSY NMR spectrum of 27 in DMSO-d_6.

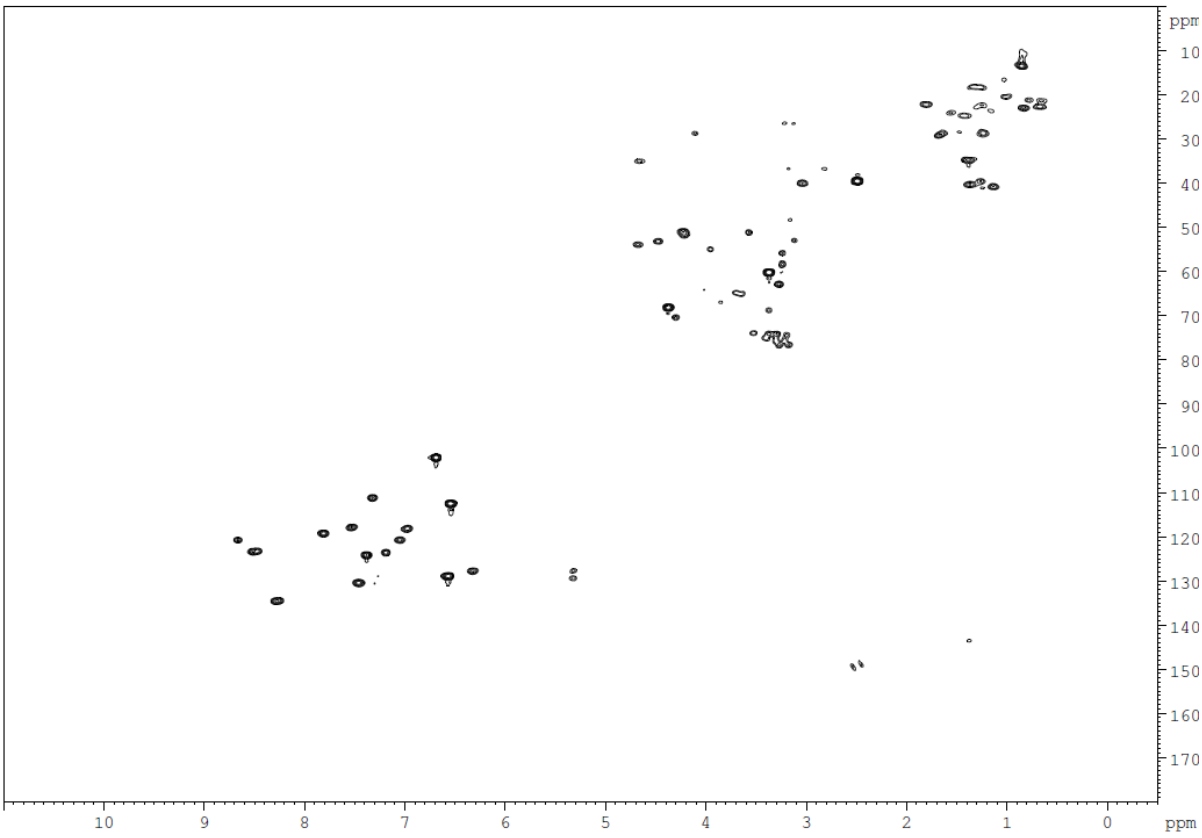


Figure S57. ^{1}H,^{13}C HSQC NMR spectrum of 27 in DMSO-d_6.

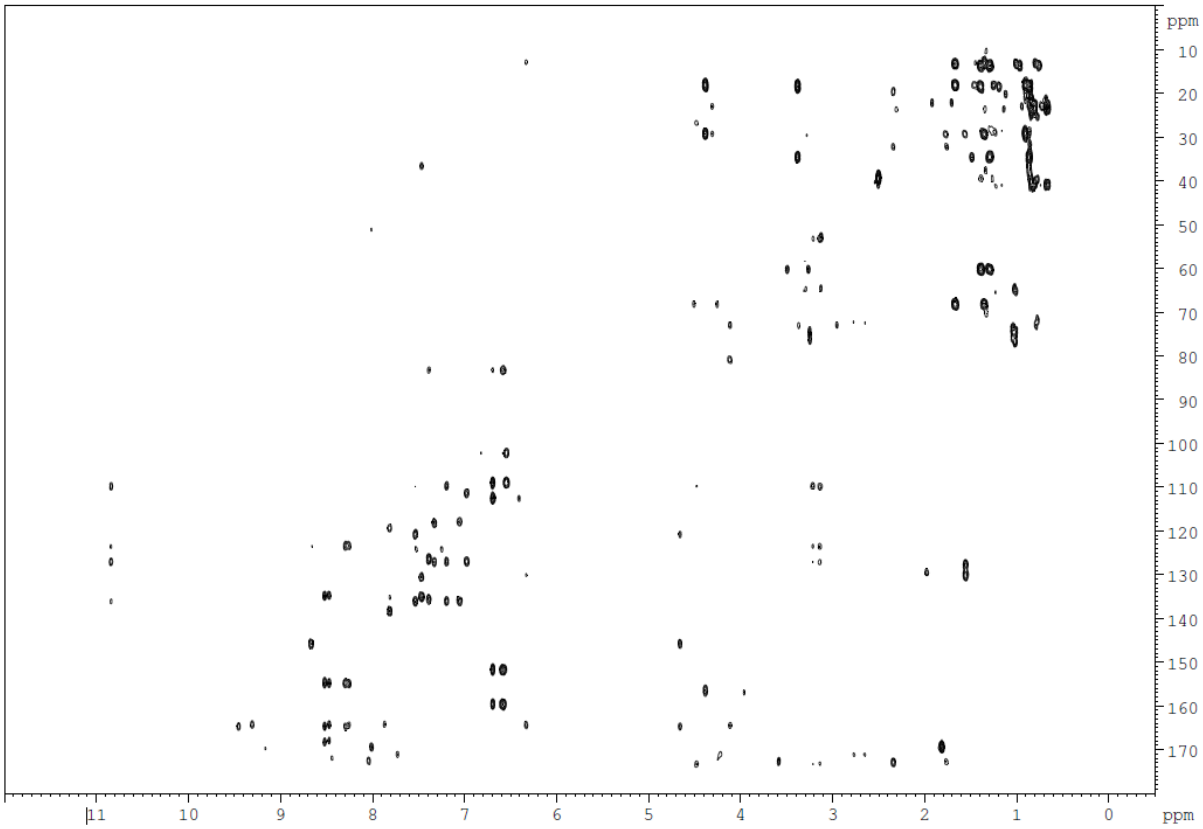


Figure S58. $^{1}H,^{13}C$ HMBC NMR spectrum of 27 in DMSO-d_6.

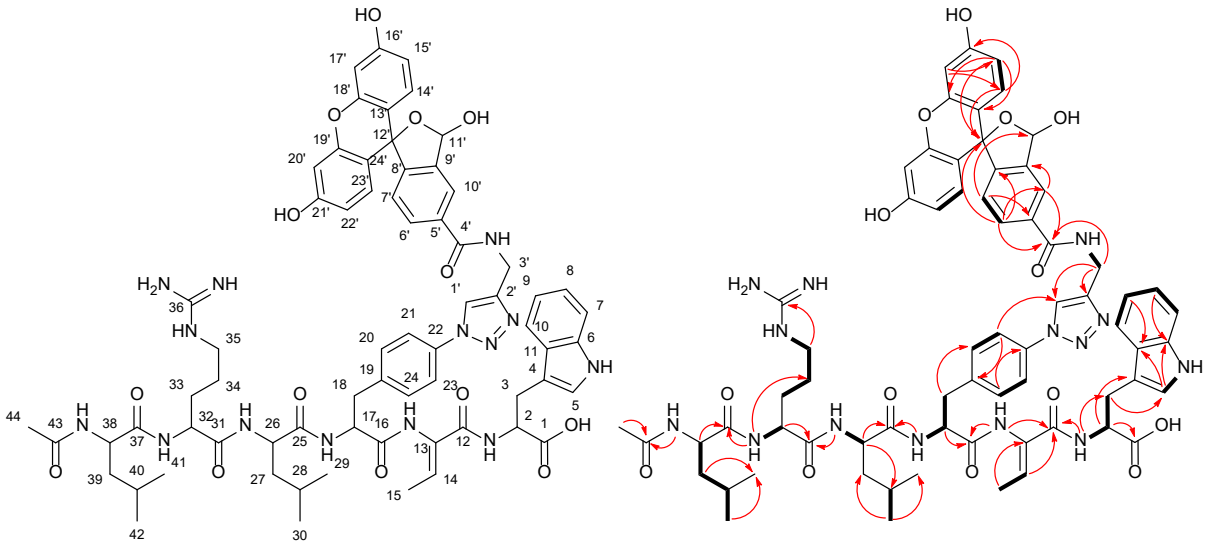


Figure S59. Carbon numbering, COSY and HMBC correlation of malpinin-5-FAM (27). COSY key correlations and HMBC key correlations are indicated as bold lines and red arrows, respectively.

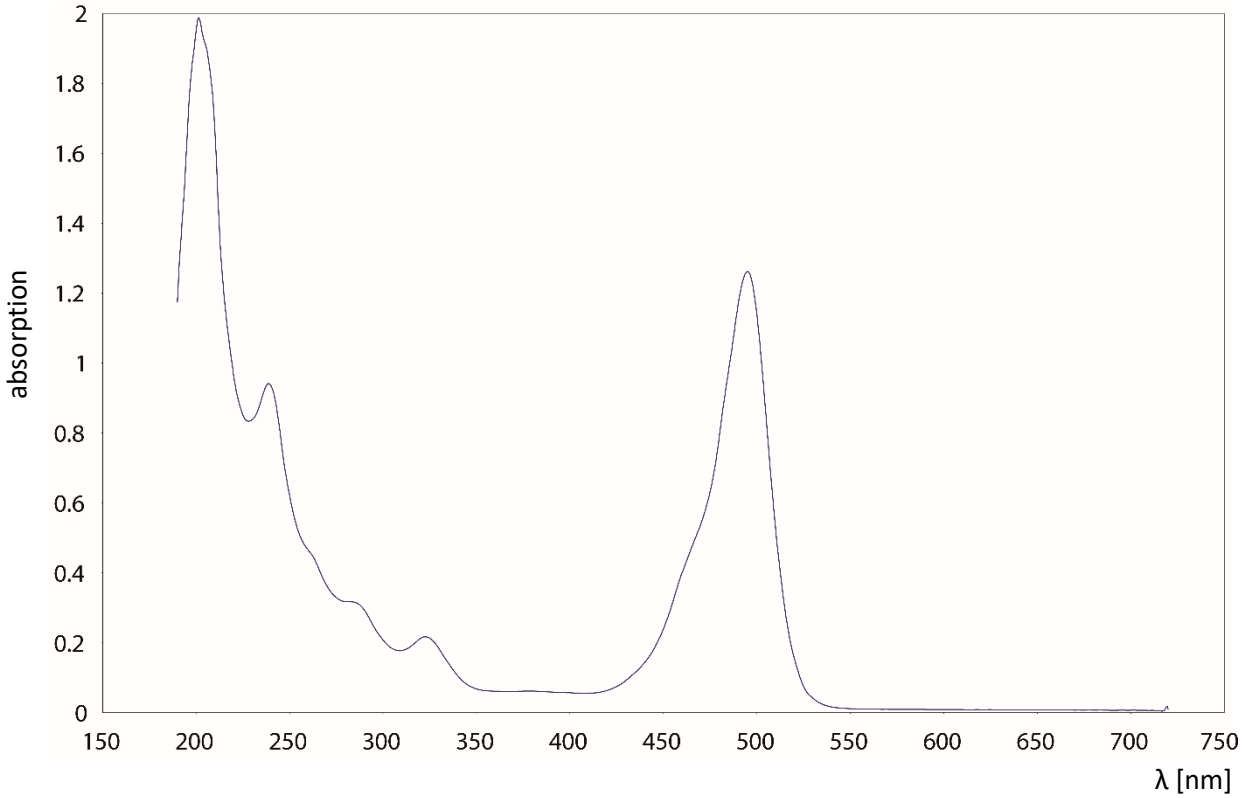


Figure S60. UV/Vis spectrum of 27 in H_2O.

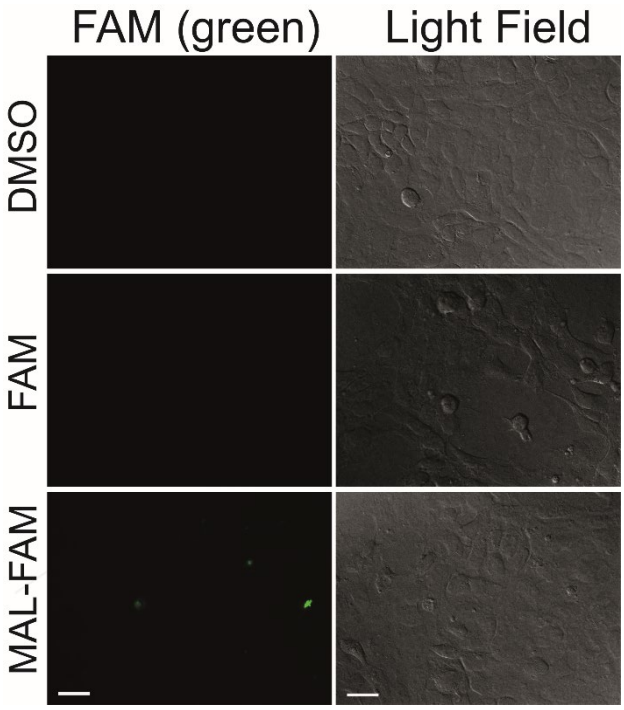


Figure S61. Fluorescence microscopy of HEK-cells. HEK-293-cells (1 x 10^6) were incubated with 30 µM fluorescein (FAM), malpinin-coupled FAM (MAL-FAM, **27**) or DMSO (0.1%) as vehicle for 1 h at 37 °C. Pictures are representative images from n = 3. Scale bar is 10 µm.

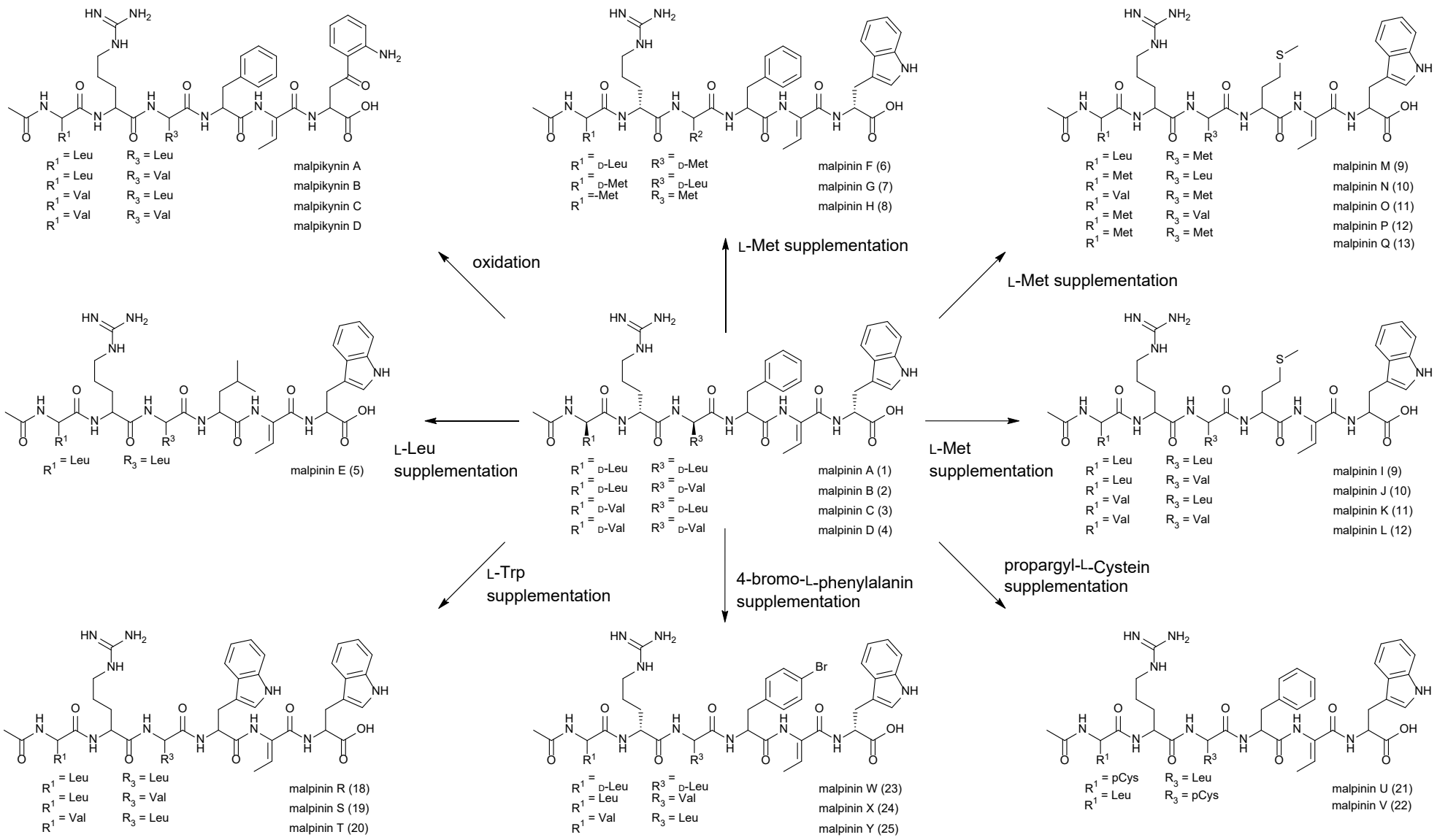


Figure S62. Malpinin A-related metabolites synthesised by MalA.

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