Fungal biosurfactants from *Mortierella alpina*

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Table of contents

Experimental procedures

Cultivation, extraction and metabolite purification

Organisms and strain identification. *M. alpina* strain ATCC32222 was obtained from the American Type Culture Collection (ATCC). All other *M. alpina* strains DH187 (= SF:6524), DH189 (= SF:9789), and DH192(= SF:2698) as well as the *M. cystojenkinii* strain DH191 (= SF:11396) were provided by the Jena Microbial Resource Collection (JMRC) and were maintained on MEP agar plates (30 g malt extract, 3 g soya peptone, 20 g agar per L) for 7 d at 25 °C prior to inoculation. Genomic DNA from all strains was isolated from 2 d old cultures grown in LB medium (180 RPM, 25 °C) as described previously.[1] Using oligonucleotides oITS1_f (5´-TCCGTAGGTGAACCTGCGG-3´) and oITS4_r (5´-TCCTCCGCTTATTGATATGC-3´) the internal transcribed spacer (ITS) spanning regions were amplified with Phusion DNA polymerase (Thermo) according to the manufacturer´s protocol. The gel-purified DNA fragments were ligated into the pJET1.2/blunt vector, sequenced and compared to the fungal genomes using NCBI BLASTn. The ITS sequence alignment and phylogenetic analysis were conducted with the MEGA 7 software.[2]

Medium optimization for malpinin production. 100 mL *Aspergillus* minimal medium[3] (AMM) containing 150 mM glucose and 35 mM NH_4NO_3 was supplemented with either L-leucine (25 mM), L-valine (25 mM), D-valine (25 mM) or was used without valine. Three replicates of the cultures were incubated at 25 °C, 120 RPM for one week. The mycelium was filtered through miracloth (Merck Millipore), resuspended in 40 mL butanol and homogenized for 5 minutes by a homogenizer (Ultra turrax, Ika). The suspension was then centrifuged (10 min, 4 °C, 3200 × g) and the resulting supernatant was evaporated to dryness. The residue was solved in 2 mL methanol and analyzed by UHPLC-MS equipped with a Zorbax Eclipse XDB-C_{18} column (50 × 2.1 mm, 1.8 µm) using a gradient of CH_3CN (eluent A) in H_2O with 0.1% FA (eluent B) with the following settings: flow:1 mL min^{-1}; temperature: 30 °C; gradient: 0-5 min: 5-100% A, 5-7 min 100% A. Chromatograms of *m/z* 859, 845 and 831 $[M + H]^+$ were extracted and the area under the curve (AUC) was calculated. AUC_{845} and AUC_{831} of the amino acid samples were normalized to the ratio of $AUC_{859,\,control}$ to $AUC_{859,\,sample}$. The factor of enhancement was calculated as $AUC_{normalized}$ to $AUC_{control}$ (Tab S1). For ^{15}N stable isotope labeling studies of **1,** 100 mL AMM containing 150 mM glucose was supplemented with 35 mM $(^{15}NH_4)_2SO_4$ or 35 mM $(^{14}NH_4)_2SO_4$. *M. alpina* ATCC 32222 was cultivated for 12 days at 25 °C prior to extraction as described above.

Cultivation, extraction and purification of 1 - 4. 15 flasks with 100 mL of optimized AMM were inoculated with *M. alpina* ATCC32222 and incubated at 25 °C, 120 RPM for one week. The mycelium was collected, resuspended in 300 mL butanol, homogenized by a homogenizer (Ultra turrax, Ika) and stirred for 2h. The suspension was then centrifuged (10 min, 4 °C, 3200 g) and the resulting supernatant was evaporated to dryness. The residue was resuspended in 2 mL of methanol/DMSO mixture (50:50). The crude extract was purified on an Agilent Infinity 1260 preparative HPLC system equipped with a Luna C_{18} column (250 × 21.2 mm, 10 µm; Phenomenex) using a gradient of CH_3CN in (eluent A) H_2O with 0.1% TFA (eluent B): flow: 20 mL min^{-1}; temperature: 20 °C; gradient: 0-1 min: 20% A, 1-21 min 20-100% A, 21-26 min 100% A. Final purification was accomplished by separation on an Agilent Infinity 1200 HPLC system equipped with a Zorbax Eclipse XDB-C_{18} column (250 × 9.4 mm, 5 µm; Agilent) using a gradient of CH_3CN (eluent A) in H_2O with 0.1% TFA (eluent B): flow: 2 mL min^{-1}; temperature: 12 °C; gradient: 0-0.5 min: 30% A, 0.5-12.5 min 30-60% A, 12.5-13 min 60-100% A, 13-15 min 100% A. The detection of malpinins was at λ = 225 nm.

Cultivation and extraction of 11 and 12. 25 flasks with 100 mL of LB medium (yeast extract 5 g, tryptone 10 g, NaCl 10 g per liter) were inoculated with *M. alpina* ATCC 32222. After two weeks of incubation at 25 °C and 120 RPM, the mycelium was collected and resuspended in a mixture of methanol, butanol and DMSO (500 mL, 12:12:1), homogenized by homogenizer (Ultra turrax, Ika) and stirred for 2 h. The suspension was then centrifuged (10 min, 20 °C, 3,200 × g) and the resulting supernatant was evaporated to dryness. The crude extract was purified on a preparative HPLC as described above with a modified gradient: 0-1 min: 5% A, 1-12.5 min 5-90% A, 12.5-13 min 90-100% A, 13-15 min 100% A. The elution of the malpibaldins was detected at λ = 205 nm.

Chemical analysis

General. LC-MS experiments were performed on an Agilent 1290 Infinity II UHPLC coupled to a 6130 Single Quadrupol mass spectrometer. MS/MS measurements were conducted using a Q Exactive Plus mass spectrometer (Thermo Scientific). NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer at 300 K. DMSO-d_6 served as solvent and internal standard (δ_H 2.49 ppm and δ_C 39.5 ppm, respectively).

Marfey's method. Marfey's method was carried out as described previously.[4] In brief, 0.25 mg of **1** - **4** were hydrolyzed in 6 N HCl (400 µL) at 150 °C for 1 h and 0.25mg of **11** and **12** were hydrolyzed in HCl (300 µL), H_2O (100 µL) and acetic acid (200 µL) at 110 °C for 18 h. After chilling the solution to ambient temperature, the hydrolysate was neutralized by adding KOH, evaporated to dryness and the residue was dissolved in 100 µL H_2O. For the derivatization reaction, 25 µL of the hydrolysate, 10 µL sodium bicarbonate (1 M) and 50 µL of a 1% (w/v) solution of Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5- L-leucine amide, L-FDLA) in acetone were mixed and incubated at 40 °C for 60 min. To stop the reaction, 10 µL 1 N HCl was added. The derivatized sample was analyzed by LC-MS using chiral FDLA amino acid standards as reference. LC-MS analyses were performed with a Luna Omega Polar C_{18} column (50×2.1 mm, 1.6 µm; Phenomenex) using a gradient of CH_3CN (eluent A) in H_2O with 0.1% FA (eluent B) with the following settings: flow: 1 mL min^{-1}; temperature: 30 °C; gradient: 0-4 min: 5-100% A, 5-6 min 100% A). The amino acid derivatives were detected by their mass ($[M + H]^+ = M_{amino\ acid} + M_{FDLA} - M_{Fluorine}$).

Detection of 1 from oil droplets. From the surface of MEP agar plates with *M. alpina*, 0.2 µL of oily droplets were diluted in 20 µl methanol and subjected to UHPLC-MS analysis. The separation was carried out on a Zorbax RRHD Eclipse Plus C_{18} column (50 × 2.1 mm, 1.8 µm; Agilent) using a gradient of CH_3CN with 0.1% FA (eluent A) in H_2O with 0.1% FA (eluent B) according to the following settings: flow: 1 mL min^{-1}; temperature: 30 °C; gradient: 0-1 min: 80-95% A, 1-1.5 min 95% A. The compounds were detected by ESI-MS in positive and negative ionization mode.

Peptide synthesis

General. Protected amino acids (Trp-O-Me, D-Phe-O-Me, D-Val-O-Me, L-Leu-N-Boc, D-Leu-N-Boc) were purchased from Carl Roth, all other reagents were purchased from Sigma-Aldrich. Water was removed by lyophilization. All reactions were performed with water-free solvent and under nitrogen atmosphere. The progress of conversion during the reaction was followed by UHPLC-MS. Intermediates were used without further purification for subsequent synthesis.

Amine deprotection. The peptide (0.125 M) and two equivalents of anisole (0.25 M) were dissolved in methylene chloride (CH_2Cl_2). Subsequently, TFA was added dropwise to make a 20% solution. The reaction was stopped after 1 to 2 hours by neutralization with KOH, extraction with H_2O and extraction with a saturated NH_4Cl solution. The organic layer was concentrated under reduced pressure.

Acid deprotection. The peptide (0.1 M) was dissolved in methanol and at least 20 equivalents of lithium hydroxide (2 M) were added (pH >11). The reaction was stopped after 3 to 18 hours by doubling the volume with H_2O and acidification with HCl to pH 4. Subsequently, methanol was removed under reduced pressure and the remaining aqueous solution was extracted three times with CH_2Cl_2. The organic extracts were pooled and concentrated.

Linear peptide coupling. To extend the linear peptide, the deprotected acid was dissolved in CH_2Cl_2 to give a solution of 0.2 M. 4 equivalents of DIPEA (*N,N*-diisopropyl ethylamine) and 1.1 equivalents of TBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate) were added and incubated 5 minutes. The peptide coupling solution was added immediately to the deprotected amines (0.2 M) in CH_2Cl_2 to give a final concentration of 0.1 M. The reaction was stopped after 3 to 18 hours by extraction with a saturated NH_4Cl solution and aqueous extraction. The organic phase was concentrate.

Macrocyclization procedure. The linear pentapeptide was deprotected as described above, starting with the acid deprotection procedure. The resulting deprotected linear peptide was either purified by HPLC (see below) or recrystallized in methanol. In a mixture of 5% DMSO in CH_2Cl_2, the linear pentapeptide and 4 equivalents of DIPEA were added (final concentration of 0.01 M). To initiate the reaction 1.1 equivalents HATU (1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*] pyridinium 3-oxid hexafluorophosphate) were added. Additional amounts of HATU were added, if educts were evident by HPLC-MS. The reaction proceeded for 3 to 18 hours. Without stopping the reaction, CH_2Cl_2 was removed under reduced pressure and additional DMSO was added to a maximal volume of 9 mL.

Purification by preparative HPLC. The cyclopeptide solution (in DMSO) was immediately subjected to an Agilent Infinity 1260 preparative HPLC system equipped with a Luna C_{18} column (250 × 21.2 mm, 10 µm; Phenomenex) using a gradient of CH_3CN (eluent A) in H_2O with 0.1% TFA (eluent B) with the following protocol: flow: 20 mL min^{-1}; temperature: 20 °C; gradient: 0-1 min: 20% A, 1-21 min 20-100% A, 21-26 min 100% A. The elution of the peptides was detected at 225 nm. The fractions containing the peptide were concentrated under reduced pressure.

Purification by semi-preparative HPLC. Purification of the linear intermediates and cyclized synthetic peptides were accomplished by separation on an Agilent Infinity 1200 HPLC system equipped with a Synergi Polar-RP column (150 × 4.6 mm,

4 µm) using a gradient of CH_3CN (eluent A) in H_2O with 0.1% TFA (eluent B) according to the following protocol: flow: 1.5 mL min^{-1}; temperature: 10 °C; gradient: 0-0.5 min: 30% A, 0.5-12.0 min 30-66.8% A, 12.0-12.5 min 66.8-100% A, 12.5-14 min 100% A). The elution of the peptides was monitored at 205 nm.

Detection by UHPLC-MS. Each reaction was followed by UHPLC-MS. The separation was carried out on a Zorbax RRHD Eclipse Plus C_{18} column (50 × 2.1 mm, 1.8 µm; Agilent) using the following gradient of CH_3CN (eluent A) in H_2O with 0.1% TFA (eluent B): flow: 1.0 mL min^{-1}; temperature: 20 °C; gradient: 0-0.5 min: 5-95% A, 0.5-1 min 95% A. Compounds of higher hydrophobicity were separated using a second protocol: flow: 1.0 mL min^{-1}; temperature: 20° C; gradient: 0-4 min: 5-72% A, 4-4.5 min 72-95% A, 4.5-5 min 95% A. Signals were detected by ESI-MS in positive and negative ionization mode.

Determination of biological activities

Bacterial growth inhibition assay. The following organisms were tested for their susceptibility to the compounds as described previously[5]: *Bacillus subtilis JMRC:STI:10880*, *Staphylococcus aureus JMRC:STI:10760*, *Escherichia coli JMRC:ST:33699* , *Pseudomonas aeruginosa* JMRC:ST:33772, *Pseudomonas aeruginosa JMRC:ST:337721*, *Staphylococcus aureus* JMRC :ST :33793 (multi-resistent), *Enterococcus faecalis* JMRC:ST:33700 (vancomycin-resistent), *Mycobacterium vaccae* JMRC :STI:10670, *Sporobolomyces salmonincolor*, JMRC:ST:35974, *Candida albicans* JMRC: STI:25000, *Penicillium notatum* JMRC:STI:50164.

Cytotoxicity assay. Cells were grown in RPMI 1640 (CAMBREX 12-167F) medium supplemented with 2 mM ultraglutamine 1 (CAMBREX 17-605E/U1), 550 µL L^{-1} gentamicin sulfate (CAMBREX 17-518Z), and 10% heat inactivated fetal bovine serum (GIBCO Life Technologies 10270-106) at 37 °C in 5% CO_2 in high density polyethylene flasks (NUNC 156340). For the cytotoxicity assay approximately 10,000 HeLa cells were seeded in 0.1 mL culture medium in microplates and incubated for 48 hours without the test substances. To test the cytotoxic effect of natural products on HeLa, the dilutions of the compounds were carried out carefully on the subconfluent monolayers of HeLa cells after the preincubation time. The cells were incubated for 72 hours at 37 °C in a humidified atmosphere and 5% CO_2. The adherent HeLa cells were fixed by glutaraldehyde (MERCK 1.04239.0250) and stained with a 0.05% solution of methylene blue (SERVA 29198) for 15 min. After gently washing, the stain was eluted by 0.2 mL of 0.33 N HCl in the wells. The optical densities were measured at 660 nm (methylene blue) in a SUNRISE microplate reader (TECAN). Under the experimental conditions, the signal from the methylene blue is proportional to the number of viable cells. The cytolytic effect of compounds were analyzed in compare to negative control. The 50% cytotoxicity concentration (CC_{50}) was defined as the test compound concentration required for destruction in 50% of the cell monolayer compared to untreated control. Four replicates were assayed.

Determination of physicochemical properties

Surface tension measurements. The surface tension of sample solutions was determined by the ring tear-off method using a De Nouy ring tensiometer (Krüss Processor Tensiometer K12, Krüss) equipped with a platinum ring (radius 9.545 mm). Stock solutions of the samples were prepared in dimethyl sulfoxide (DMSO, RotiSolv®, Carl Roth) and serially diluted in concentrations ranging from 1.95 µg mL^{-1} – 500 µg mL^{-1} in 10% (v/v) DMSO/water mixture. 10 mL of each sample was filled in a tempered 43 mL glass vessel (SV10) for measurements at 25 °C. The density of the solvent mixture was set to 1.011 g cm^{-3}.[6] Additionally, the detergent sodium dodecyl sulfate (SDS, Carl Roth) was used as control in the same concentration range and measured analogous. Each sample and concentration were measured in triplicates. Data were reported as mean ± standard deviation. The surface tension vs. concentration plots were extrapolated to estimate the critical micelle concentration (CMC).[7]

Phase segregation in O/W emulsions. 25 µL of olive oil was suspended in 9.975 mL of water by ultra-sonication for 5 min. This emulsion was added to the substances und ultraonicated again for 2 minutes to prepare the following solutions: 1 (0.1 mg mL^{-1}), Tween 80 (1 mg mL^{-1}), SDS (2 mg mL^{-1}) and water (served as reference). The slope of the phase segregation was monitored at λ = 650 nm with a UV/VIS photometer for up to 4 hours. Relative phase segregation was determined:

$$\textbf{\textit{relative phase segregation}}\ [\%] = \frac{\textbf{\textit{slope}}\ (\textbf{\textit{detergent}})}{\textbf{\textit{slope}}\ (\textbf{\textit{water}})}\ \textbf{\textit{x}}\ \mathbf{100\%}$$

Calculation of HLB values: Hydrophilic-lipophilic balance (HLB) values have been calculated according to Griffin´s method.[8]

Quantities and physical properties of isolated metabolites and synthesized compounds

Isolated metabolites

Malpinin A (**1**), 37.7 mg, white greyish amorphous powder, UV (CH_3CN) λ_{max} 224 nm, 280 nm; HR-ESI-MS data, see Table S1; ^1H and ^{13}C NMR spectroscopic data, see Table S4.

Malpinin B (**2**), 15.6 mg, white greyish amorphous powder, UV (CH_3CN) λ_{max} 224 nm, 280 nm; HR-ESI-MS data, see Table S1; ^1H and ^{13}C NMR spectroscopic data, see Table S4.

Malpinin C (**3**), 3.0 mg, white greyish amorphous powder, UV (CH_3CN) λ_{max} 224 nm, 280 nm; HR-ESI-MS data, see Table S1; ^1H and ^{13}C NMR spectroscopic data, see Table S4.

Malpinin D (**4**), 6.0 mg, white greyish amorphous powder, UV (CH_3CN) λ_{max} 224 nm, 280 nm; HR-ESI-MS data, see Table S1; ^1H and ^{13}C NMR spectroscopic data, see Table S4.

Malpibaldin A (**11**), 7.5 mg, white yellowish amorphous powder, UV (CH_3CN) λ_{max} 202 nm; HR-ESI-MS data, see Table S1; ^1H and ^{13}C NMR spectroscopic data, see Table S6.

Malpibaldin B (**12**), 3.6 mg, white yellowish amorphous powder, UV (CH_3CN) λ_{max} 218 nm, 282 nm; HR-ESI-MS data, see Table S1; ^1H and ^{13}C NMR spectroscopic data, see Table S6.

Malpikynin A-E (**6-10**) and *malpibaldin C* (**13**) were detected as accompanying compounds by UPLC-MS (Fig. S2), HR-ESI-MS (Table S1, Fig. S51-S52) and ^1H NMR spectroscopy (Table S5) and could not be isolated in quantifiable amounts.

Synthesized compounds

Malpibaldin A (**11**), cyclo-(D-Val-L-Leu-D-Phe-D-Leu-L-Leu), 1.7 mg, white yellowish amorphous powder, UV (CH_3CN) λ_{max} 202 nm; HR-ESI-MS data, see Table S1; ^1H NMR spectroscopic data, see Fig. S50.

Malpibaldin B (**12**), cyclo-(D-Val-L-Leu-D-Trp-D-Leu-L-Leu), 1.0 mg, white yellowish amorphous powder, UV (CH_3CN) λ_{max} 218 nm, 282 nm; HR-ESI-MS data, see Table S1; ^1H NMR spectroscopic data, see Fig. S50.

Malpibaldin B stereoisomer a (**12a**), cyclo-(D-Val-L-Leu-D-Trp-L-Leu-D-Leu), 1.1 mg, white yellowish amorphous powder, UV (CH_3CN) λ_{max} 218 nm, 282 nm; ^1H NMR spectroscopic data, see Fig. S50.

Malpibaldin B stereoisomer b (**12b**), cyclo-(D-Val-D-Leu-D-Trp-L-Leu-L-Leu), 1.6 mg, white yellowish amorphous powder, UV (CH_3CN) λ_{max} 218 nm, 282 nm; ^1H NMR spectroscopic data, see Fig. S50.

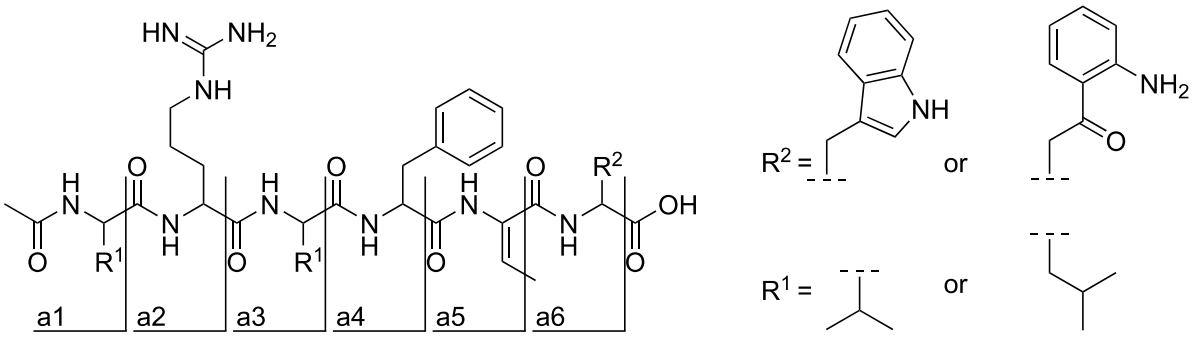
Table S1. HR-ESI-MS-data of derivatives of malpinins, malpikynins and malpibaldins. DOU = Degree of unsaturation.

compound	no.	$[M+H]^+$	chem. formula of M	DOU
Malpinin A	**1**	859.4827	$C_{44}H_{62}N_{10}O_8$	19
Malpinin B	**2**	845.4665	$C_{43}H_{60}N_{10}O_8$	19
Malpinin C	**3**	845.4674	$C_{43}H_{60}N_{10}O_8$	19
Malpinin D	**4**	831.4515	$C_{42}H_{58}N_{10}O_8$	19
Malpinin E	**5**	825.4971	$C_{41}H_{65}N_{10}O_8$	14.5
Malpikynin A	**6**	863.4777	$C_{43}H_{62}N_{10}O_9$	18
Malpikynin B	**7**	849.4641	$C_{42}H_{60}N_{10}O_9$	18
Malpikynin C	**8**	849.4635	$C_{42}H_{60}N_{10}O_9$	18
Malpikynin D	**9**	835.4475	$C_{41}H_{58}N_{10}O_9$	18
Malpikynin E	**10**	829.4950	$C_{40}H_{65}N_{10}O_9$	13.5
Malpibaldin A	**11**	586.3963	$C_{32}H_{51}N_5O_5$	9.5
Malpibaldin B	**12**	625.4062	$C_{34}H_{52}N_6O_5$	11.5
Malpibaldin C	**13**	602.3912	$C_{32}H_{51}N_5O_6$	9.5

Table S2. Optimization of AMM by supplementation with 25 mM valine.

	control	**L-valine**			**D-valine**		
$[M+H]^+$	AUC [mAu min]	AUC [mAu min]	$AUC_{normalized}$ [mAu min]	Factor	AUC [mAu min]	$AUC_{normalized}$ [mAu min]	Factor
859	6.99 E + 07	4.89 E + 07	6.99 E + 07	1.00	3.23 E + 07	6.99 E + 07	1.00
845	1.94 E + 07	2.79 E + 07	3.99 E + 07	2.06	9.59 E + 06	2.08 E + 07	1.07
831	3.32 E + 06	1.05 E + 07	1.50 E + 07	4.52	1.34 E + 06	2.90 E + 06	0.87

Table S3. ESI-MS-MS data of **1** – **4** and **6** – **9.**



	fragments *m/z* $[M+H]^+$					
compound	a1	a2	a3	a4	[a5+2H]	a6
malpinin A (**1**)	128.11	284.21	397.29	544.36	629.41	813.47
malpinin B (**2**)	128.11	284.21	383.28	530.35	615.40	799.46
malpinin C (**3**)	114.09	270.19	383.28	530.35	615.40	799.46
malpinin D (**4**)	114.09	270.19	369.26	516.33	599.37	785.45
malpikynin A (**6**)	128.11	284.21	397.29	544.36	629.41	813.47
malpikynin B (**7**)	128.11	284.21	383.28	530.35	615.40	799.46
malpikynin C (**8**)	114.09	270.19	383.28	530.35	615.40	803.47
malpikynin D (**9**)	114.09	270.19	369.26	516.33	603.38	789.43

Table S4. NMR data of **1** – **4** in DMSO-d_6. *signals overlapping.

	malpinin A (1)		**malpinin B (2)**		**malpinin C (3)**		**malpinin D (4)**	
pos.	δ_C [ppm]	δ_H [ppm], M (*J* [Hz])	δ_C [ppm]	δ_H [ppm], M (*J* [Hz])	δ_C [ppm]	δ_H [ppm], M (*J* [Hz])	δ_C [ppm]	δ_H [ppm], M (*J* [Hz])
	D-tryptophan							
1	173.3		173.3		173.3		173.3	
2-NH		7.81, d (7.6)		7.84, d (7.5)		7.83, d (7.6)		7.84, d (7.6)
2	53.5	4.46, dt (5.3, 12.1)	53.4	4.46, dt (5.3, 12.0)	53.4	4.45, m (5.5)	53.4	4.46, m
3	26.9	a: 3.11, m*	26.9	a: 3.11, m*	26.8	a: 3.12, m*	26.8	a: 3.11, m*
		b: 3.21, dd (5.2, 14.8)		b: 3.21, dd (5.0, 14.6)		b: 3.21, dd (5.1, 14.6)		b: 3.21, dd (5.0, 14.6)
4	109.9		109.9		109.9		109.9	
5	123.8	7.19, d (2.6)	123.7	7.18, d (3.7)	123.8	7.18, m*	123.8	7.18, m*
5-NH		10.84, d (1.8)		10.84, d (1.4)		10.83, d (1.4)		10.82, d (1.5)
6	127.1		127.1		127.1		127.1	
7	118.1	7.52, d (8.0)	118.1	7.52, d (7.9)	118.1	7.52, d (7.8)	118.1	7.52, d (8.0)
8	118.4	6.97, t (7.0)	118.3	6.97, t (7.4)	118.4	6.97, t (7.4)	118.4	6.97, t (7.5)
9	120.9	7.05, t (7.1)	120.9	7.05, t (7.5)	120.9	7.05, t*	120.9	7.05, t (8.1)
10	111.4	7.32, d (8.0)	111.4	7.32, d (8.1)	111.4	7.32, d (8.1)	111.4	7.32, d (8.1)
11	136.1		136.1		136.1		136.1	
	dehydrobutyric acid							
12	164.3		164.3		164.3		164.3	
13-NH		9.11, s		9.16, s		9.11, s		9.16, s
13	130.2		130.2		130.2		130.2	
14	128.2	6.32, q (7.0)	128.0	6.31, q (7.0)	128.0	6.31, q (7.1)	128.1	6.31, q (7.1)
15	13.1	1.52, d (7.1)	13.1	1.51, d (7.0)	13.1	1.52, d (7.1)	13.1	1.51, d (7.1)
	L-phenylalanine							
16	170.2		170.2		170.2		170.2	
17-NH		8.35, d (8.0)		8.35, d (8.0)		8.36, d (8.0)		8.35, d (7.9)
17	54.3	4.58, m (8.0, 10.7)	54.2	4.65, m (4.6, 10.9)	54.2	4.58, m	54.2	4.64, m
18	37.2	a:2.77, dd (10.8, 13.6)	37.4	a:2.74, dd* (11.0, 13.5)	37.3	a:2.75, dd (11.0, 13.5)	37.3	a:2.74, dd (11.0, 13.4)
		b:3.11, m*		b:3.08, m*		b:3.09, m*		b:3.09, m*
19	137.8		137.8		137.8		137.7	
20-24	129.3	7.26, d (7.5)	129.2	7.27, d (7.3)	129.2	7.26, d* (7.3)	129.2	7.27, d* (7.5)
21-23	128.0	7.23, t (7.5)	128.0	7.22, t (7.5)	128.0	7.23, t* (7.5)	128.0	7.23, t* (7.6)
22	126.3	7.17, t (7.2)	126.3	7.16, t (7.2)	126.2	7.17, t* (7.2)	126.3	7.16, t* (7.5)
	D-leucine		D-valine		D-leucine		D-valine	
25	172.0		170.8		172.0		170.8	
26-NH		7.73, d (7.8)		7.50, d (8.8)		7.77, d (7.7)		7.57, d (9.1)
26	51.1	4.22, m*	57.2	4.16, dd (8.6,6.0)	51.1	4.23, m*	57.4	4.15, m
27	41.0	1.16, m	30.8	1.75, m	41.1	1.14, m	30.7	1.73, m
28	23.8	1.23, m*	18.9	0.56, d (6.7)	23.8	1.22, m*	18.9	0.54, d (6.7)
29	22.9	0.72, d (6.7)	17.3	0.50, d (6.7)	22.8	0.71, d* (6.5)	17.4	0.51, d (6.8)
29'	21.7	0.70, d (6.4)			21.7	0.69, d* (6.5)		
	D-arginine							
30	171.0		171.0		171.0		171.1	
31-NH		8.04, d (7.7)		8.09, d (7.9)		8.01, d (7.8)		8.06, d (8.2)
31	51.9	4.21, m*	51.9	4.26, m*	51.9	4.22, m*	52.1	4.27, m
32	28.5	a:1.48, m*	28.4	a:1.47, m*	28.7	a:1.45, m*	28.7	a:1.47, m*
		b:1.63, m*		b:1.63, m		b:1.60, m		b:1.61, m
33	25.0	a:1.39, m*	25.0	a:1.39, m*	25.0	a:1.38, m	25.0	a:1.39, m
		b:1.45, m*		b:1.44, m*		b:1.43, m*		b:1.45, m*
34	40.4	3.03, dt (5.9, 11.8)	40.4	3.03, m*	40.4	3.03, m	40.1	3.04, m
34-NH		7.54, t (5.7)		7.41, m		7.44, m		7.45, m
35	156.7		156.6		156.6		156.6	
	D-leucine		D-leucine		D-valine		D-valine	
36	172.5		172.5		171.3		171.3	
37-NH		8.02, d (7.6)		8.00, d (7.8)		7.89, d (8.3)		7.88, d (8.5)
37	51.2	4.23, m*	51.0	4.24, m*	57.9	4.09, m	57.8	4.11, m
38	40.6	a: 1.37, m*	40.8	a: 1.38, m*	30.3	1.88, m	30.3	1.88, m
		b: 1.41, m*		b: 1.41, m*				
39	24.1	1.57, m	24.1	1.56, m	19.1	0.81, d* (3.5)	19.2	0.81, d (6.3)
40	23.0	0.84, d (6.7)	23.0	0.84, d (6.6)	18.2	0.80, d* (3.5)	18.2	0.79, d (6.3)
40'	21.6	0.80, d (6.6)	21.6	0.80, d (6.5)				

	acetic acid							
41	169.4		169.3		169.5		169.4	
42	22.4	1.81, s	22.4	1.80, s	22.5	1.84, s	22.4	1.83, s

Table S5. NMR data of kynurenine moieties of **6** - **9** in DMSO-d_6. *signals overlapping.

	malpikynin A (6)				**malpikynin B (7)**			
pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])	COSY (^{1}H→^{1}H)	HMBC (^{1}H→^{13}C)	δ_C [ppm]	δ_H [ppm], M (J [Hz])	COSY (^{1}H→^{1}H)	HMBC (^{1}H→^{13}C)
	D-kynurenine				D-kynurenine			
1	173.2				n.d.			
2-NH		7.83, d*	2			7.82, d*	2	
2	48.4	4.79, m	2-NH, 3	3, 4	48.1	4.79, m	2-NH	
3	40.2	a:3.40, m* b:3.44, m*	2	1, 2, 4	39.7	a:3.39, m* b:3.44, m*		4
4	198.5				198.3			
5	116.3				116.1			
6	151.4				151.3			
6-NH2		n.d.				n.d.		
7	116.6	6.75, d (8.4)	8	5, 9	116.4	6.74, d (8.3)	8	5, 9
8	134.3	7.25, m*	7, 9	6, 10	134.3	7.25, m*	7, 9	6, 10
9	114.5	6.53, t (7.5)	8, 10	5, 7	114.5	6.53, t (7.5)	8, 10	5, 7
10	130.9	7.71, d*	9	4, 6, 8	131.1	7.72, d*	9	4, 6, 8

	malpikynin C (8)				**malpikynin D (9)**			
pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])	COSY (^{1}H→^{1}H)	HMBC (^{1}H→^{13}C)	δ_C [ppm]	δ_H [ppm], M (J [Hz])	COSY (^{1}H→^{1}H)	HMBC (^{1}H→^{13}C)
	D-kynurenine				D-kynurenine			
1	173.0				172.9			
2-NH		7.84, d*	2			7.82, d*	2	
2	48.2	4.79, m	2-NH, 3	1, 4	48.2	4.79, m	2-NH, 3	1
3	40.2	a:3.40, m* b:3.46, m*	2	1, 2, 4	40.2	a:3.38, m* b:3.47, m*	2	1, 2, 4
4	198.4				198.4			
5	115.8				115.9			
6	151.2				151.1			
6-NH2		n.d.				n.d.		
7	116.6	6.74, d (8.2)	8	5, 9	116.4	6.74, d (8.5)	8	5, 9
8	134.3	7.25, m*	7, 9	6, 10	134.3	7.25, m*	7, 9	6
9	114.5	6.53, t (7.4)	8, 10	5, 7	114.5	6.53, t (7.8)	8, 10	5, 7
10	131.2	7.72, d (8.7)	9	4, 6, 8	131.0	7.72, d (8.9)	9	4, 6, 8

Table S6. NMR data of **11** and **12** in DMSO-d_6. *overlapping.

	malpibaldin A (11)			**malpibaldin B (12)**	
pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])	pos.	δ_C [ppm]	δ_H [ppm], M (J [Hz])
	L-leucine			L-leucine	
1	171.5	8.84, d (7.6)	1	171.4	
2-NH		4.34, m	2-NH		7.84, d (7.5)
2	50.1	8.84, d (7.6)	2	50.1	4.46, dt (5.3, 12.0)
3	36.5	a:1.44, m*	3	36.6	a: 3.11, m*
		b:1.50, m*			b: 3.21, dd (5.0, 14.6)
4	24.1	1.54, m*	4	24.1	1.55, m*
5	22.9	0.86, d (6.5)	5	22.8	0.86, d (6.5)
6	21.5	0.77, d (6.4)	6	21.5	0.78, d (6.4)
	D-leucine			D-leucine	
7	170.8		7	170.9	
8-NH		4.29, m	8-NH		7.21, d (7.1)
8	52.0	7.26, d (7.0)	8	51.9	4.30, m
9	40.3	a: 1.39, m*	9	40.4	a: 1.39, m*
		b: 1.64, m			b: 1.62, m*
10	25.0	1.38, m*	10	24.9	1.38, m*
11	23.2	0.84, d (6.0)	11	23.2	0.84, d (6.1)
12	22.8	0.88, d (6.3)	12	22.3	0.88, d (6.1)
	D-phenylalanine			D-tryptophan	
13	170.8		13	171.2	
14-NH		8.82, d (8.0)	14-NH		8.71, d (8.2)
14	55.9	4.21, ddd (3.3, 8.6, 11.9)	14	55.3	4.23, m
15	36.7	a:2.74, dd (12.1, 13.6)	15	27.1	a: 2.90, dd (3.1, 14.4)
		b:3.09, dd (3.4, 13.8)			b: 3.19, dd (11.3, 14.5)
16	138.4		16	110.5	
17/21	128.9	7.22, m*	17	123.7	7.12, d (1.9)
18/20	128.1	7.22, m*	17-NH		10.79, d (1.4)
19	126.2	7.16, m	18	126.9	
			19	118.1	7.51, d(7.9)
			20	118.3	6.95, t (7.5)
			21	120.8	7.03, t (7.2)
			22	111.3	7.30, d (8.0)
			23	136.2	
	L-leucine			L-leucine	
22	171.7		24	171.8	
23-NH		8.49, d (6.6)	25-NH		8.45, d (6.9)
23	52.1	4.07, dt (6.5, 9.5)	25	51.9	4.12, m*
24	38.9	a: 1.13, m	26	38.8	a: 1.18, m
		b: 1.30, m			b: 1.20, m
25	23.8	0.87, m*	27	23.9	0.99, m (6.7)
26	22.8	0.62, d (6.6)	28	22.4	0.62, d (6.6)
27	21.9	0.70, d (6.5)	29	22.0	0.72, d (6.5)
	D-valine			D-valine	
28	171.6		30	171.5	
29-NH		7.57, d (9.2)	31-NH		7.59, d (9.2)
29	57.1	4.12, dd (6.8, 9.1)	31	57.2	4.11, m*
30	30.7	1.80, m (6.7)	32	30.7	1.79, m (6.7)
31	19.2	0.79, d (6.6)	33	19.1	0.81, d (6.7)
32	17.9	0.81, d (6.7)	34	18.0	0.79, d (6.7)

Table S7. Determination of the absolute configuration of amino acids by Marfey's method. A. Results for 1 - 4 compared to authentic standards. B. Results for 11 and 12 compared to authentic standards. Retention times of D- and L-amino acids are highlighted in blue and red, respectively.

A

	retention time of derivatized amino acids [min]				
sample	Leu	Val	Arg	Phe	Kyn
malpinin A (**1**)	2.66	-	1.62	2.39	2.50
malpinin B (**2**)	2.67	2.52	1.63	2.40	2.52
malpinin C (**3**)	2.66	2.51	1.63	2.39	2.51
malpinin D (**4**)	-	2.51	1.63	2.39	2.51
L-Standard	2.31	2.19	1.68	2.37	2.29
D-Standard	2.65	2.50	1.60	2.55	2.49

B

	retention time of derivatized amino acids [min]			
sample	Leu (ratio)	Val	Phe	Kyn
malpibaldin A (**11**)	2.31 + 2.61 (2:1)	2.47	2.55	-
malpibaldin B (**12**)	2.32 + 2.62 (2:1)	2.48	-	2.47
L-Standard	2.31	2.19	2.35	2.29
D-Standard	2.61	2.45	2.55	2.46

Table S8. Antiproliferative and cytotoxic activity of **1 - 4** and **11** and **12**. Compounds have been tested in a range of 1.5-50 µg mL^{-1}.

compound	**antiproliferative effect**		**cytotoxicity**	**solubility**
	HUVEC	K-562	HeLa	
	GI50 [µg mL^{-1}]	GI50 [µg mL^{-1}]	CC50 [µg mL^{-1}]	
malpinin A (**1**)	>50	>50	>50	DMSO
malpinin B (**2**)	>50	>50	>50	DMSO
malpinin C (**3**)	>50	>50	42.8 (± 0.7)	DMSO
malpinin D (**4**)	>50	>50	>50	DMSO
malpibaldin A (**11**)	>50	>50	>50	DMSO
malpibaldin B (**12**)	>50	>50	>50	DMSO

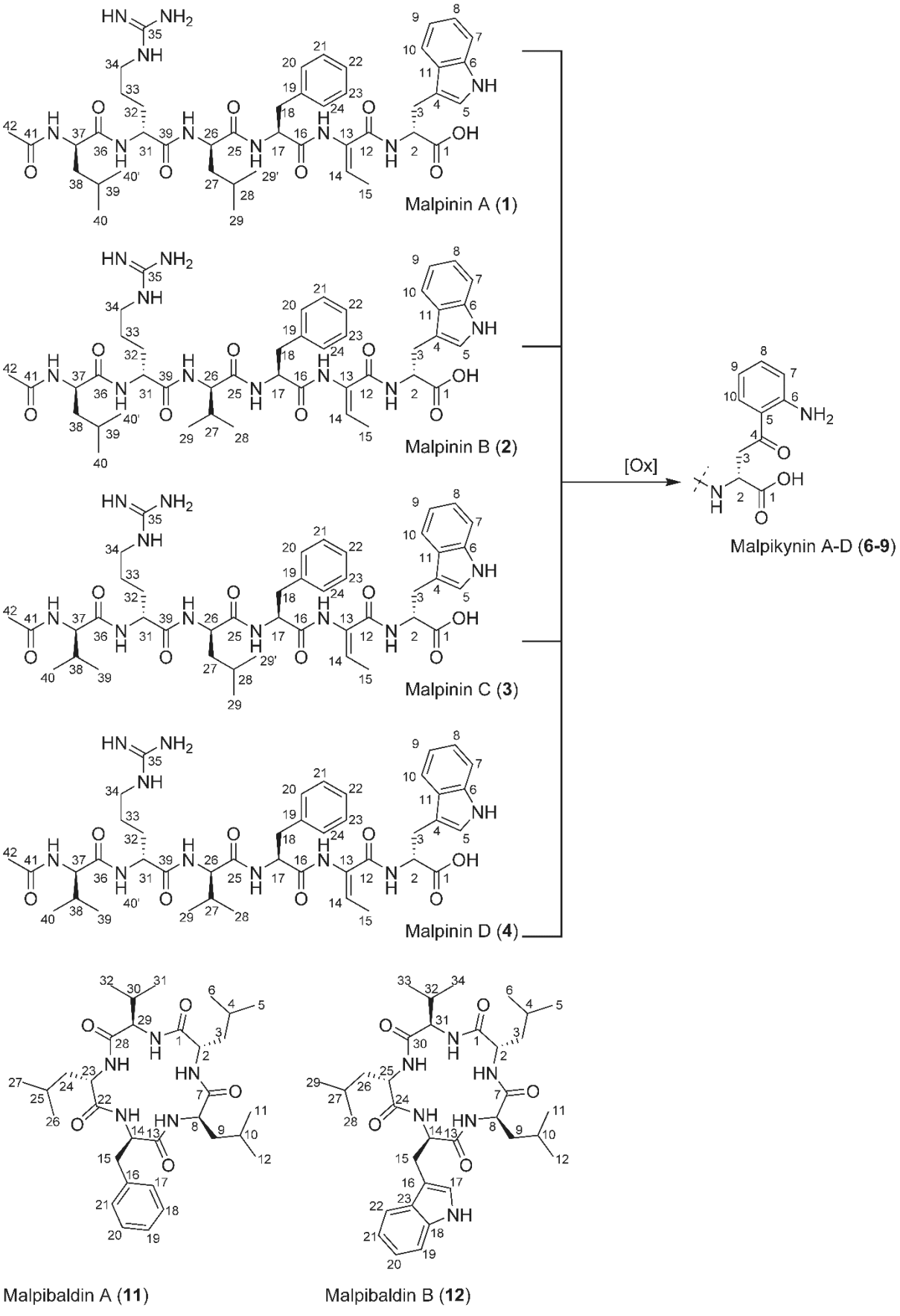


Figure S1. Numbering of carbon atoms in compounds **1 - 4, 6 - 9** and **11 - 12.**

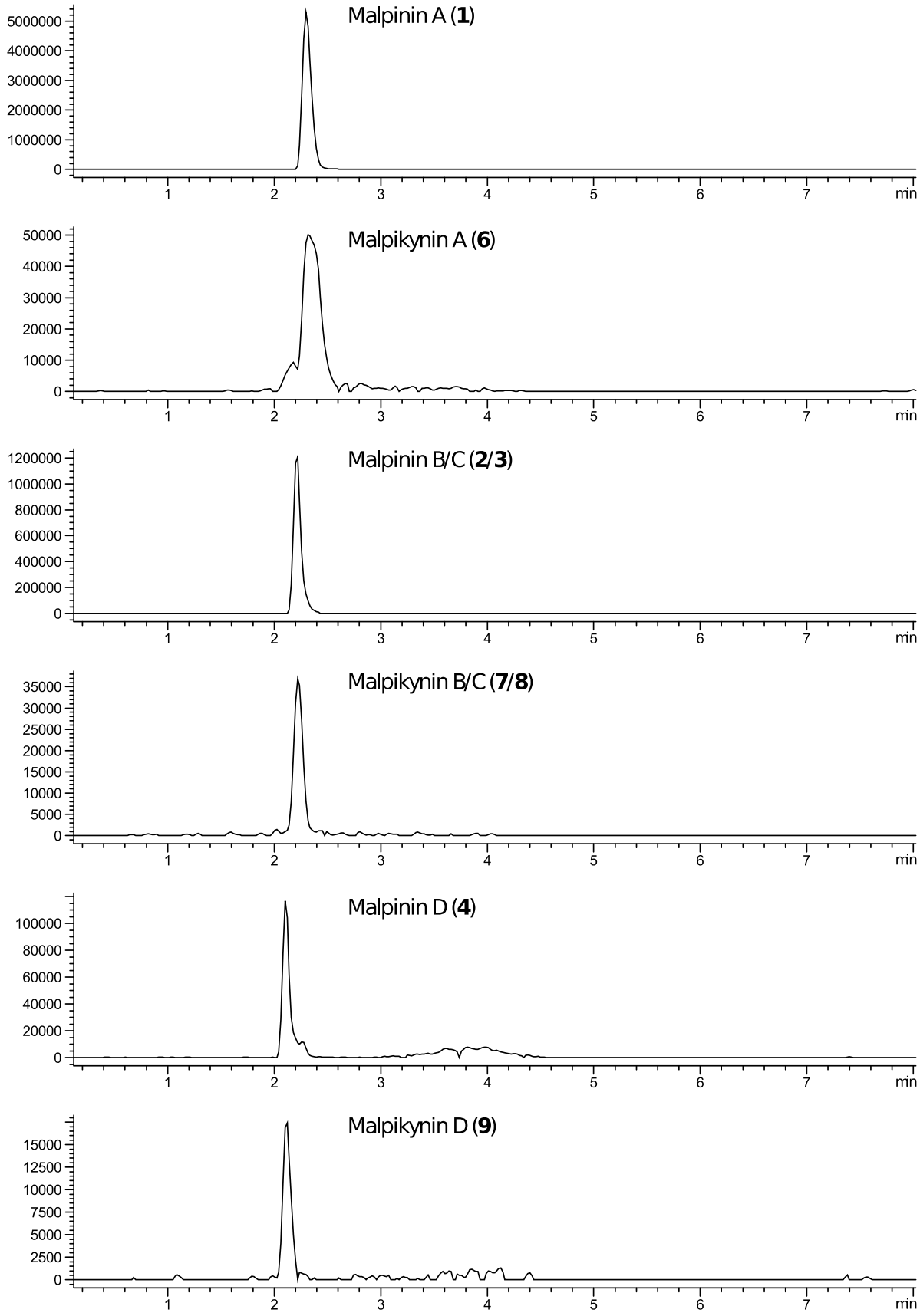


Figure S2. Abundance of malpinins **(1 – 4)** and malpikynins **(6 - 9)** in the butanolic crude extracts of *M. alpina*. EIC detected in positive mode are shown for **1** (m/z 859[M + H]$^+$), **2/3** (m/z 845[M + H]$^+$), **4** (m/z 831 [M + H]$^+$), **6** (m/z 863 [M + H]$^+$), **7/8** (m/z 849 [M + H]$^+$) and **9** (m/z 835 [M + H]$^+$). Note, that malpikynins are detectable in trace amounts (1-2%) when compared to malpinins..

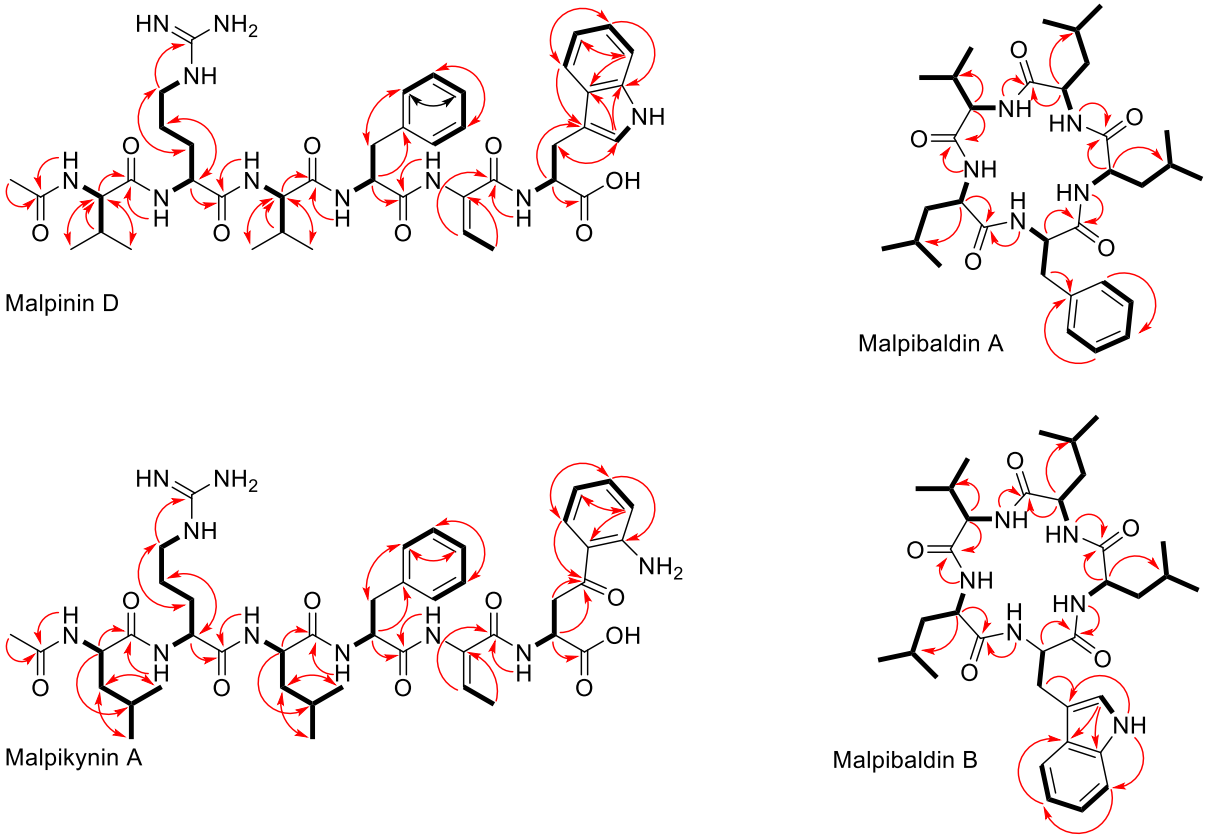


Figure S3. COSY (bold lines) and selected HMBC (red arrows) key correlations in **4** (two valine-residues), **6** (two leucine-residues), **11** and **12**.

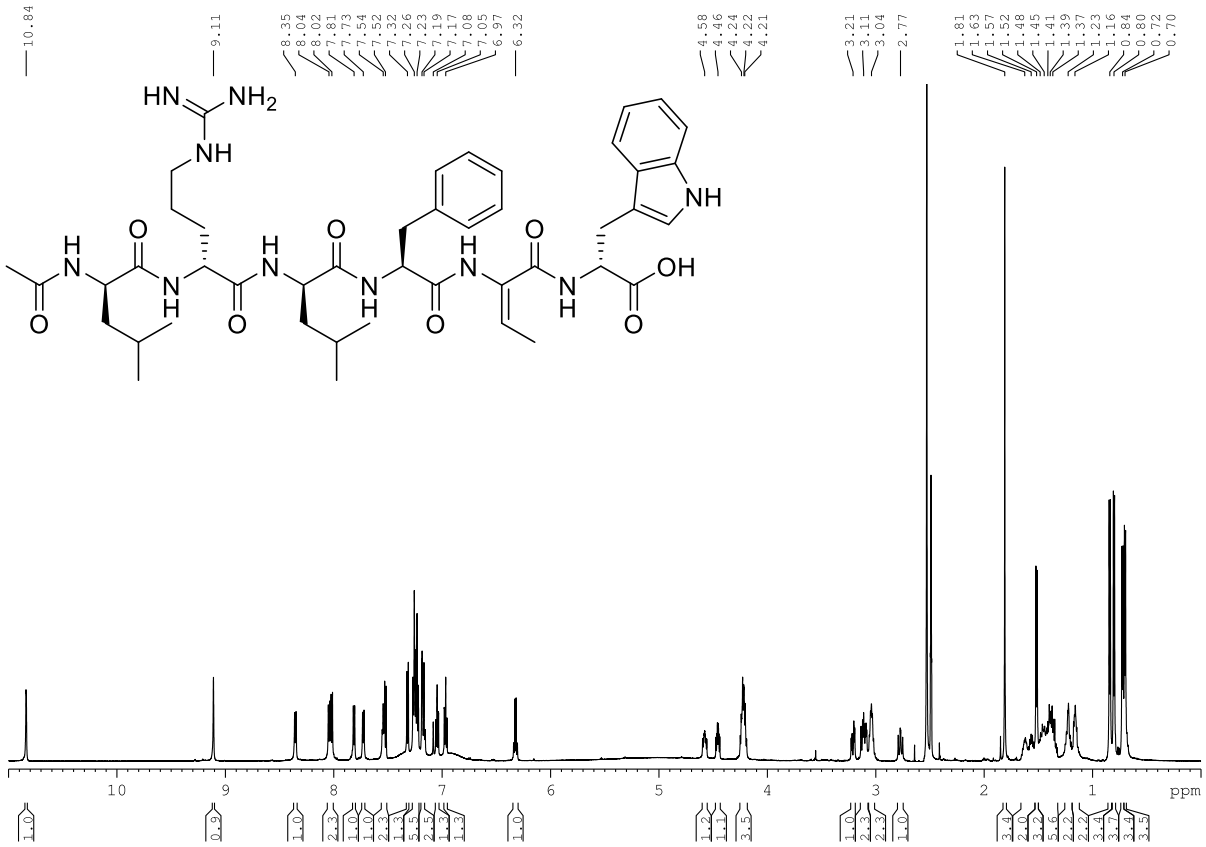


Figure S4. [1]H NMR spectrum of **1** in DMSO-d_6

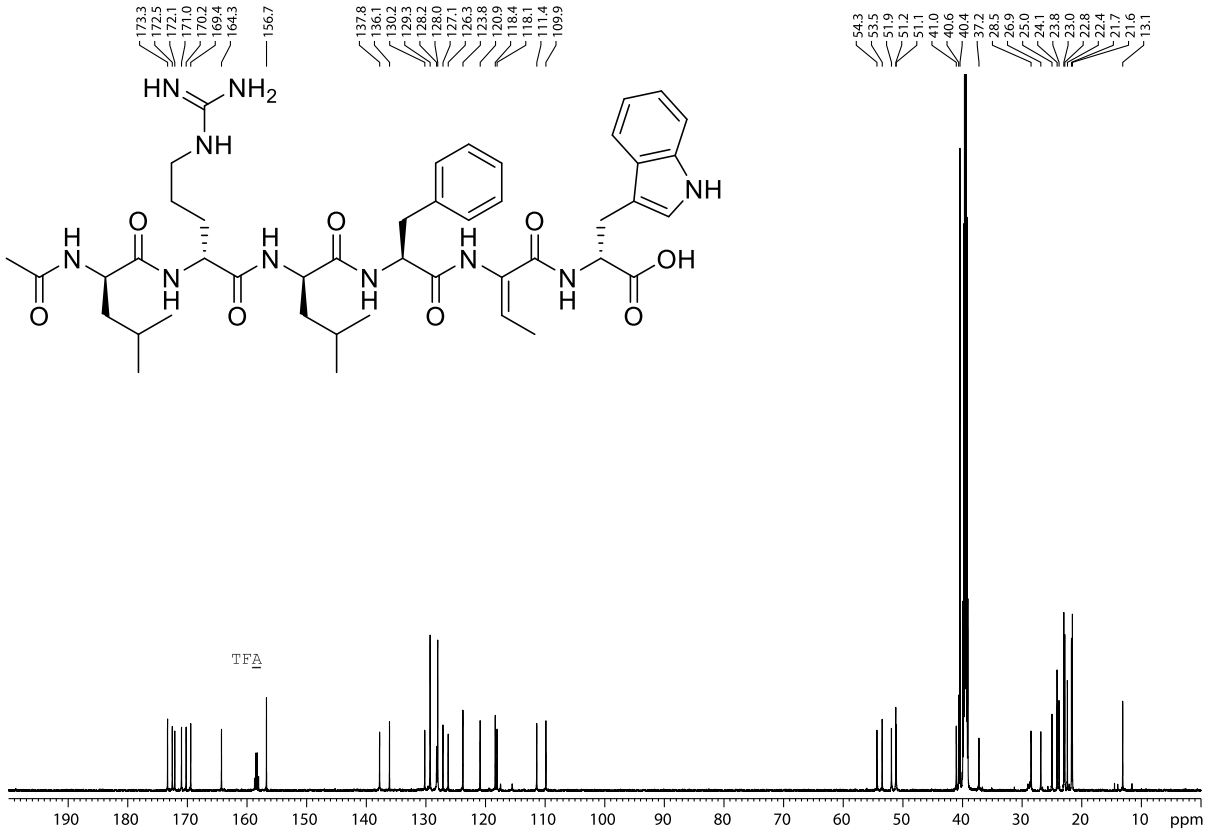


Figure S5. [1]H decoupled [13]C NMR spectrum of **1** in DMSO-d_6

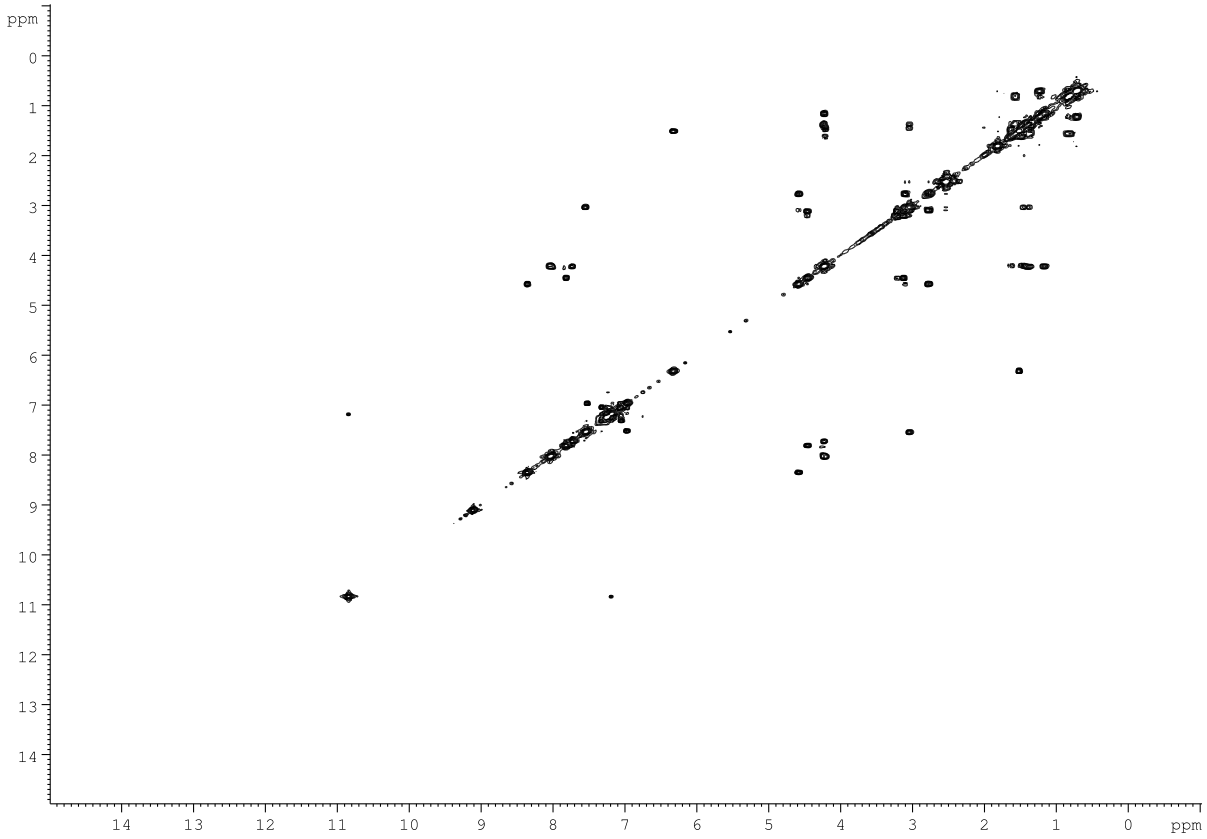


Figure S6. ^{1}H, ^{1}H COSY spectrum of **1** in DMSO-d_6

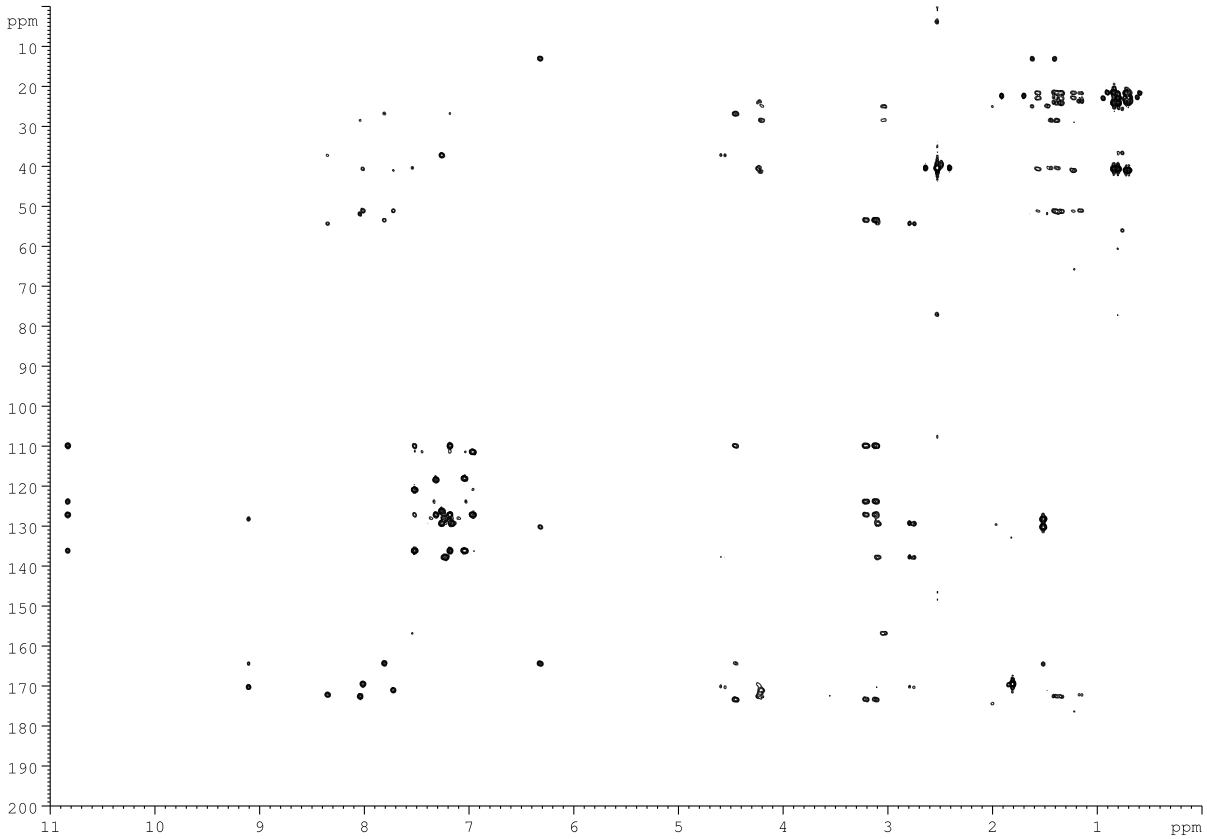


Figure S7. ^{1}H, ^{13}C HMBC spectrum of **1** in DMSO-d_6

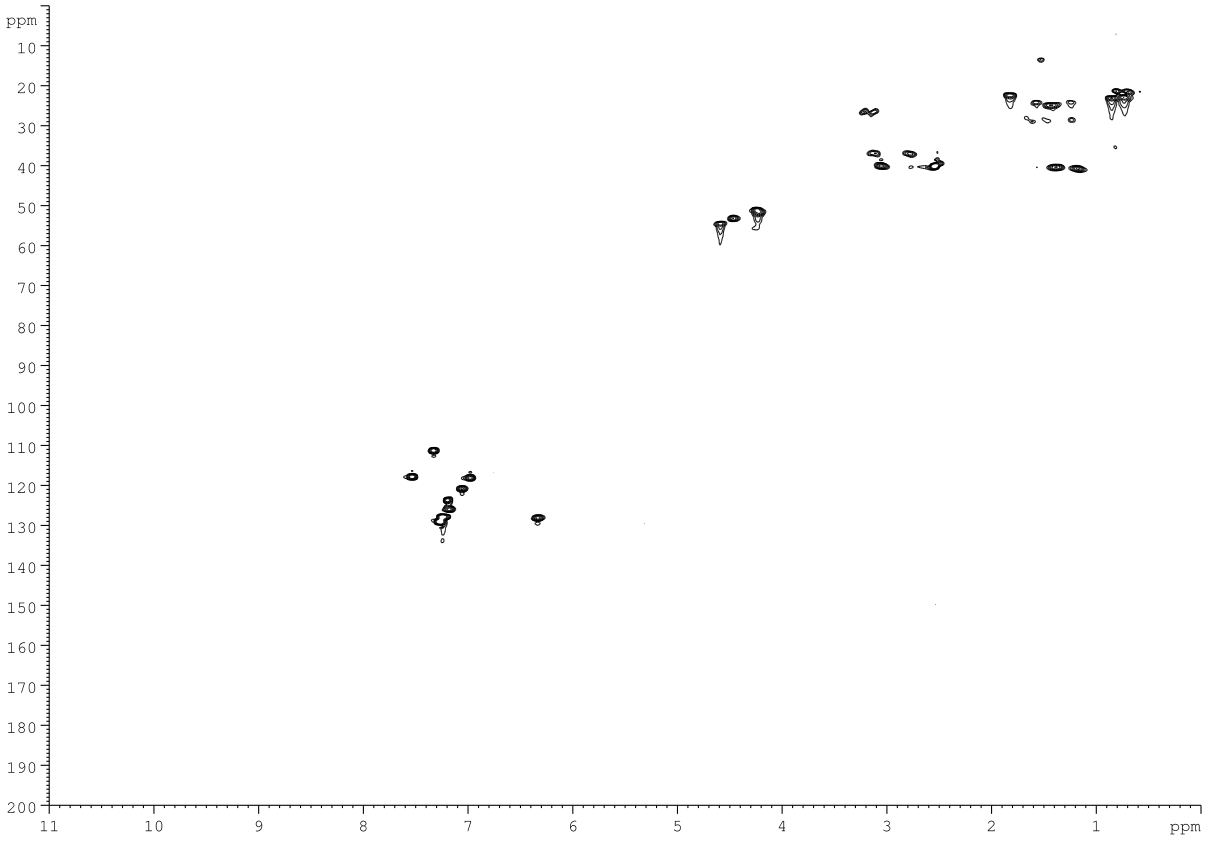


Figure S8. ^{1}H, ^{13}C HSQC spectrum of **1** in DMSO-d_6

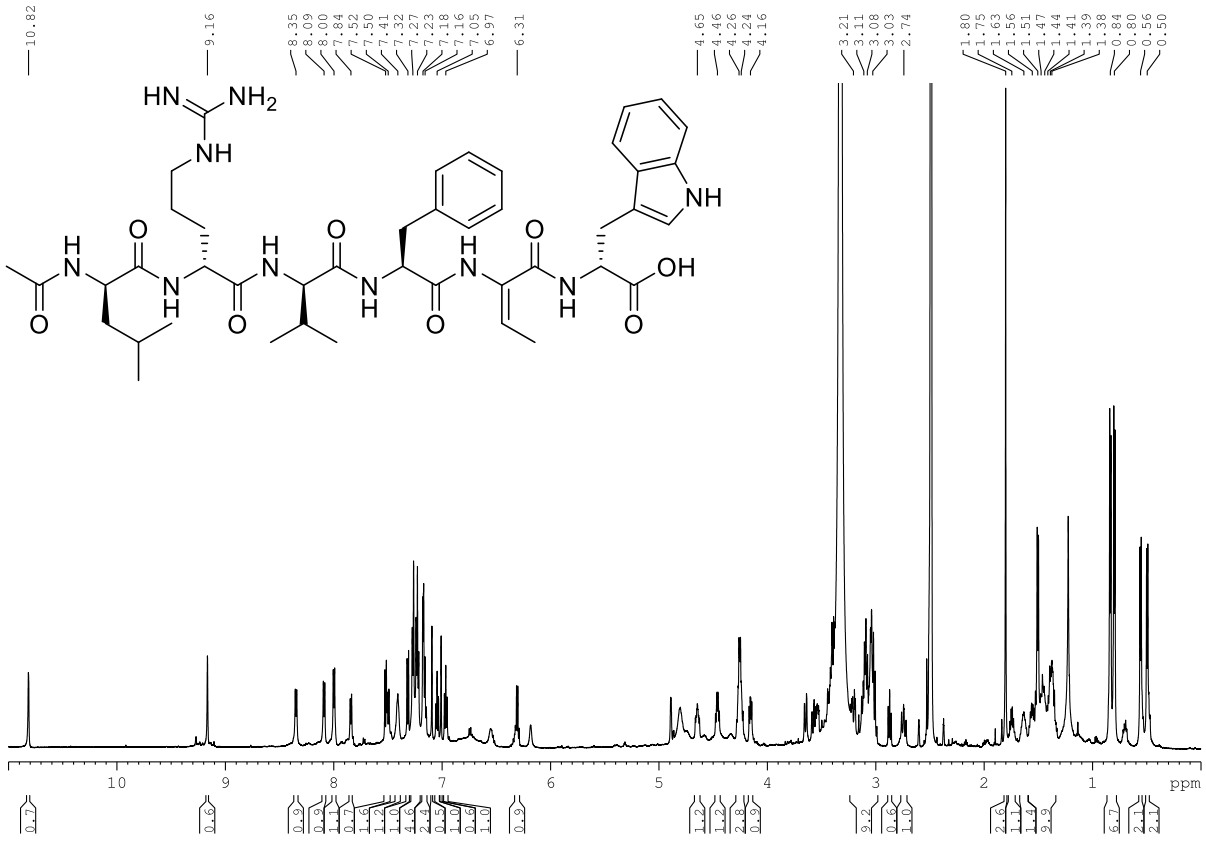


Figure S9. ^{1}H NMR spectrum of **2** in DMSO-d_6

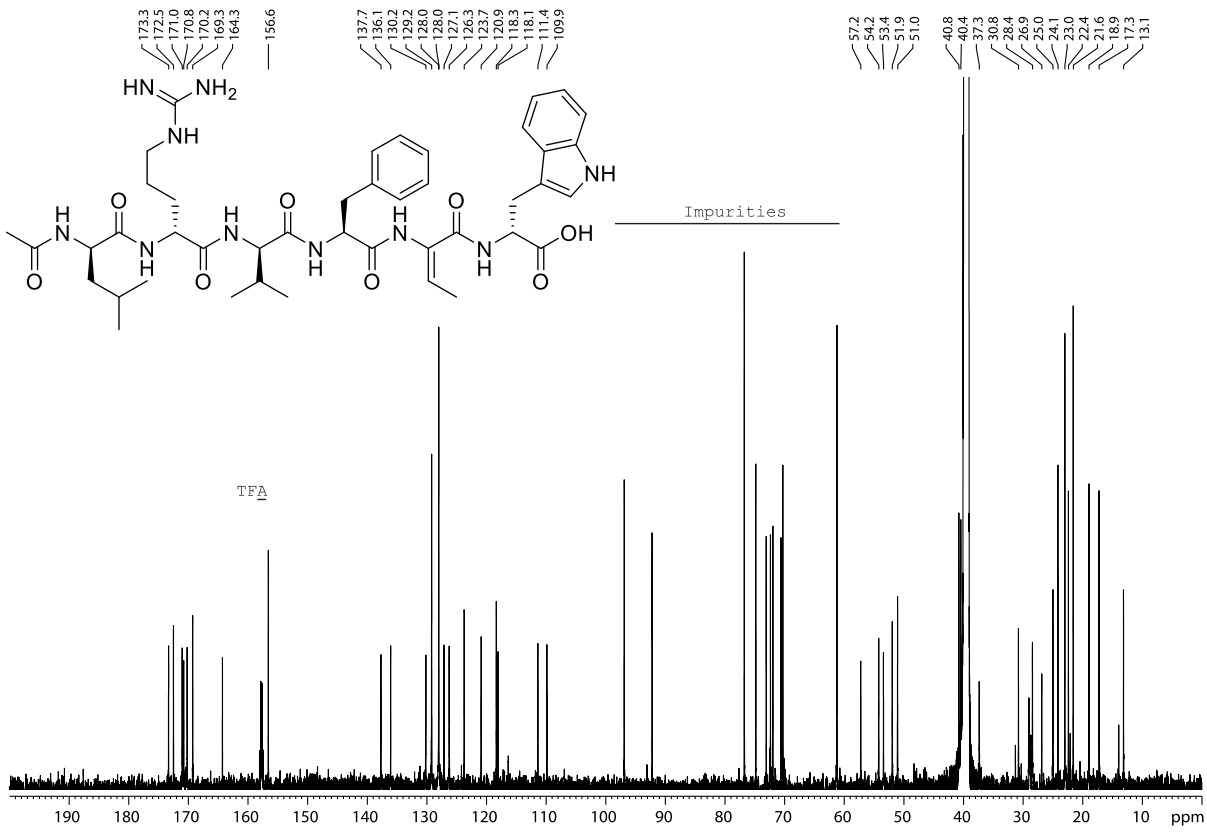


Figure S10. ^{1}H decoupled ^{13}C NMR spectrum of **2** in DMSO-d_6

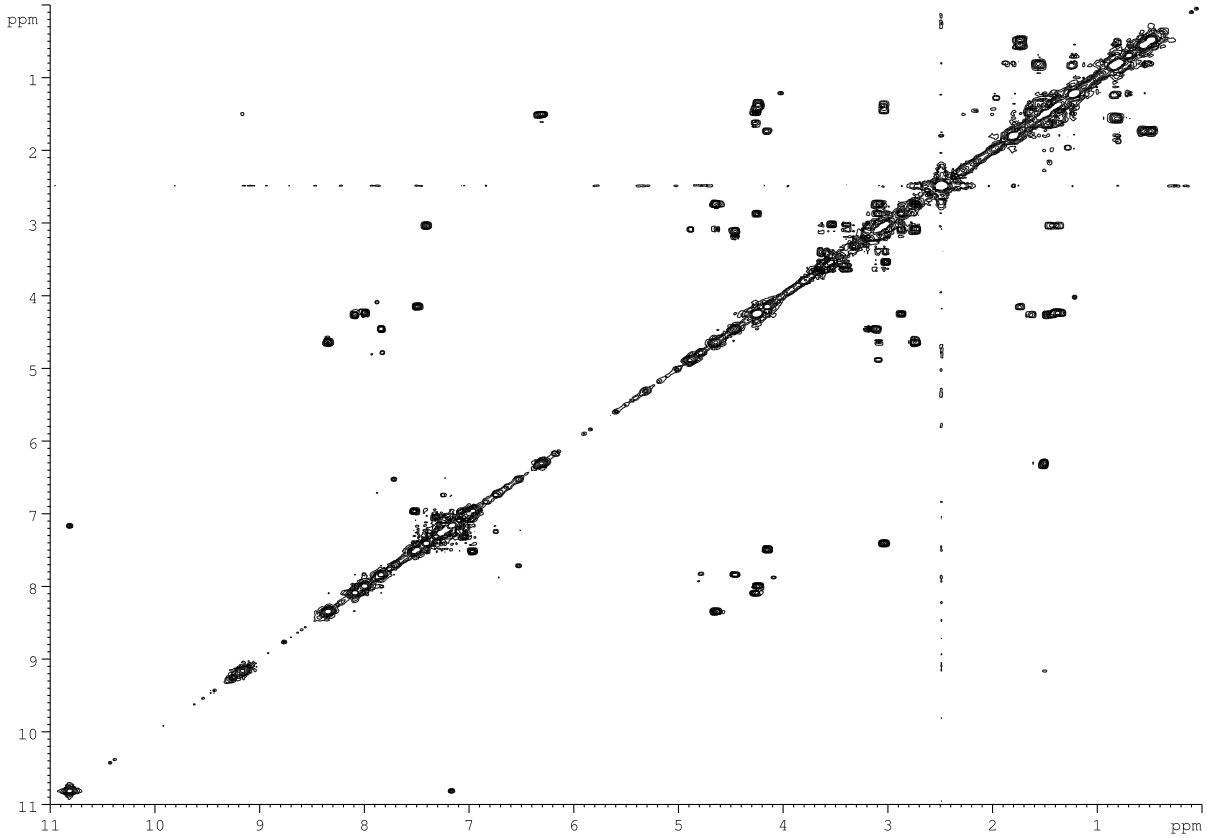


Figure S11. ^{1}H, ^{1}H COSY spectrum of **2** in DMSO-d_6

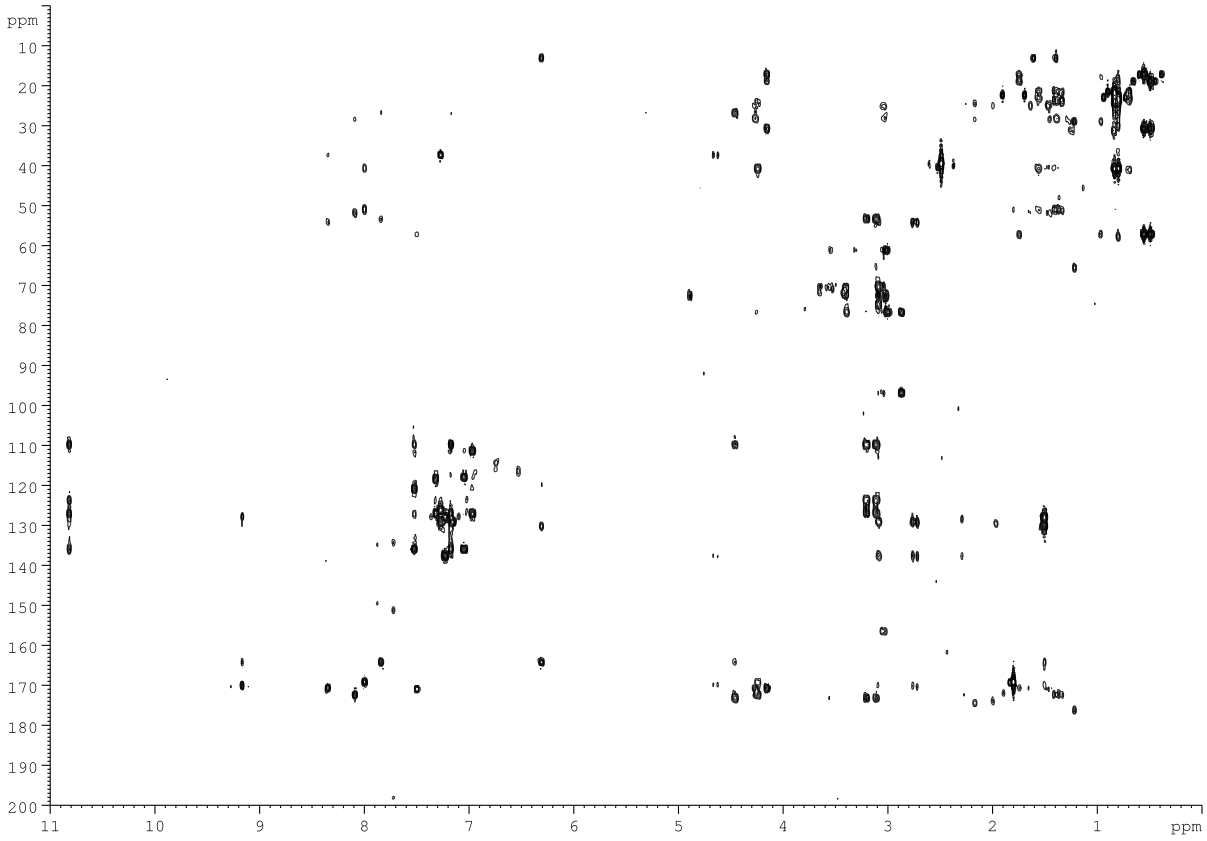


Figure S12. ^{1}H, ^{13}C HMBC spectrum of **2** in DMSO-d_6

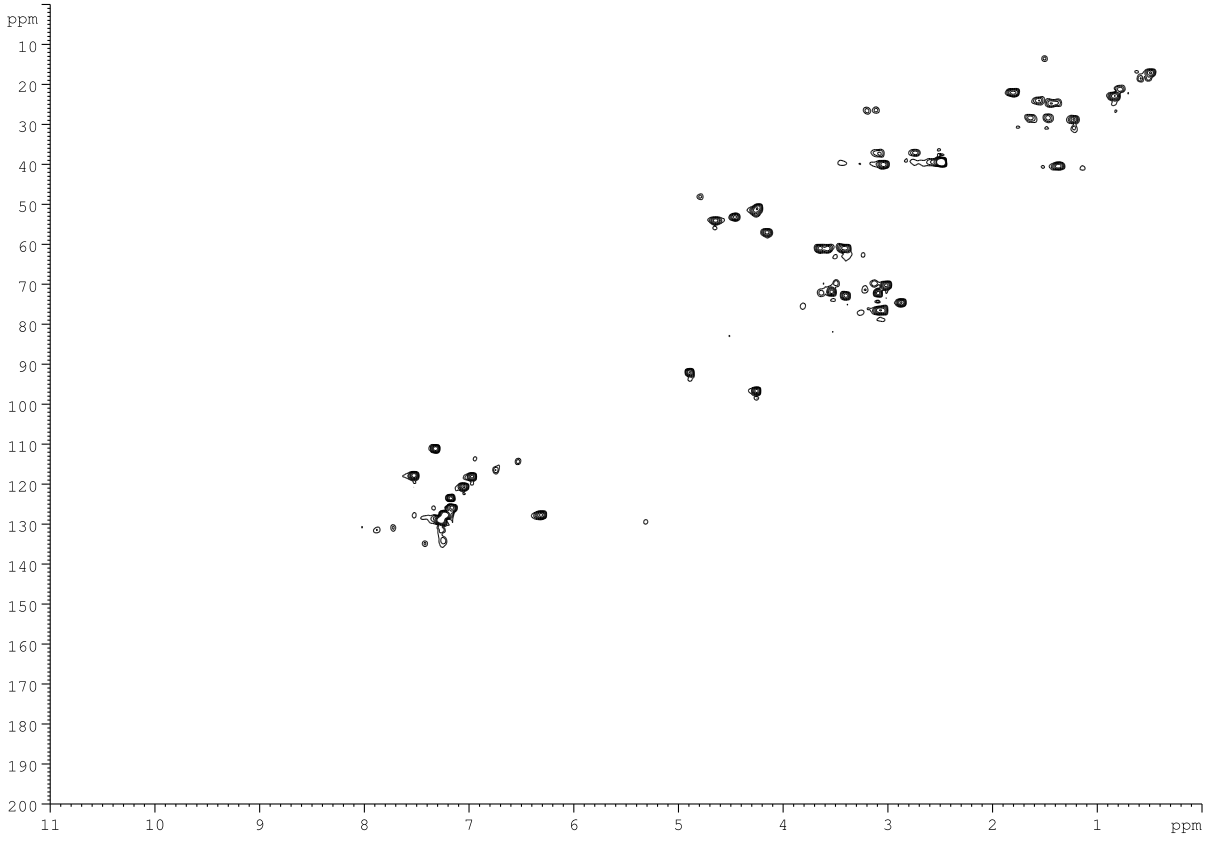


Figure S13. ^{1}H, ^{13}C HSQC spectrum of **2** in DMSO-d_6

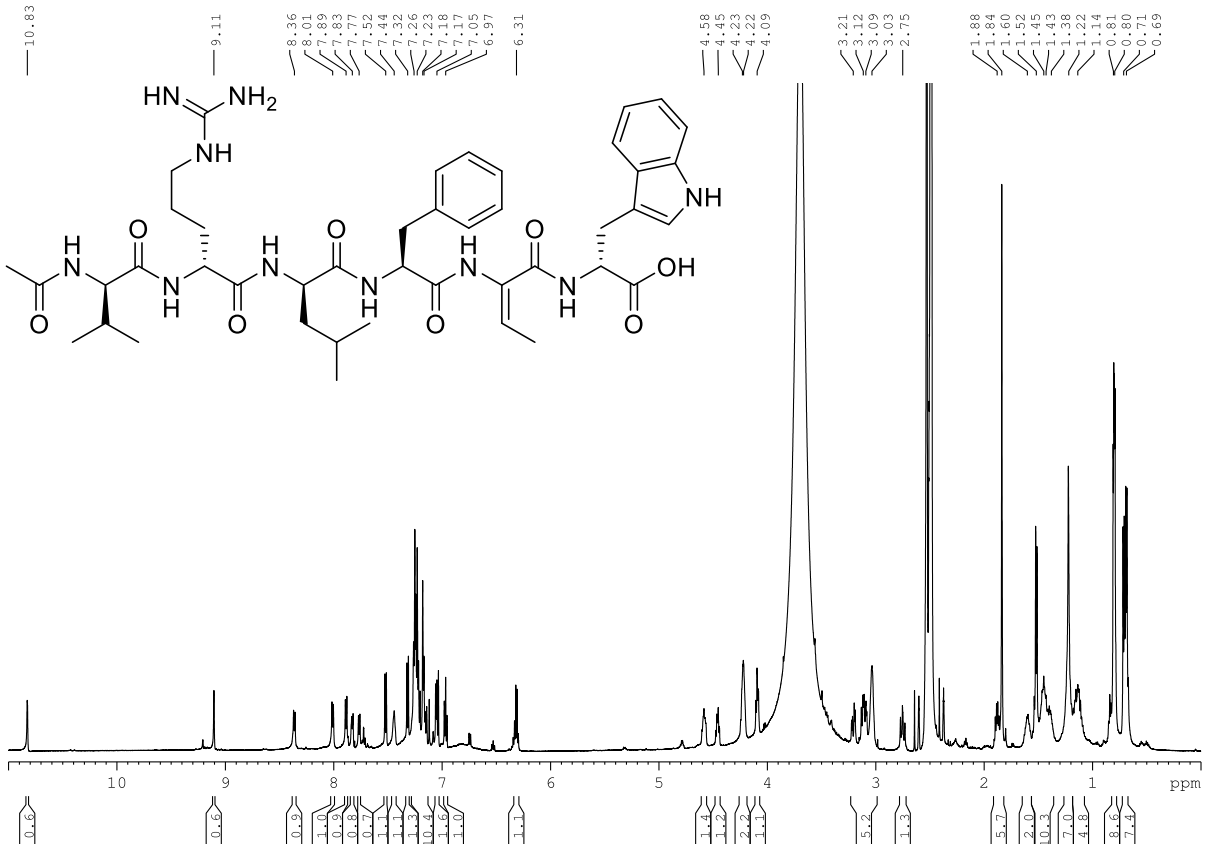


Figure S14. [1]H NMR spectrum of **3** in DMSO-*d*6

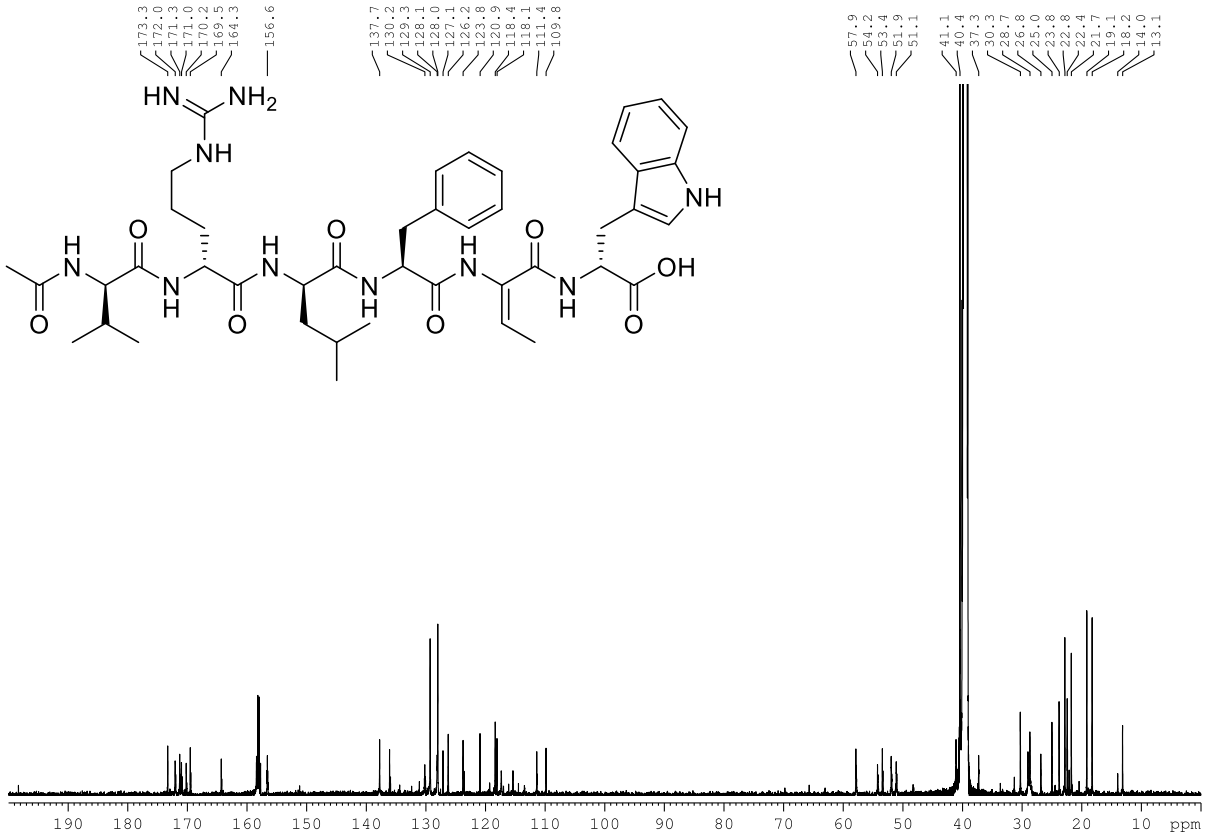


Figure S15. [1]H decoupled [13]C NMR spectrum of **3** in DMSO-*d*6

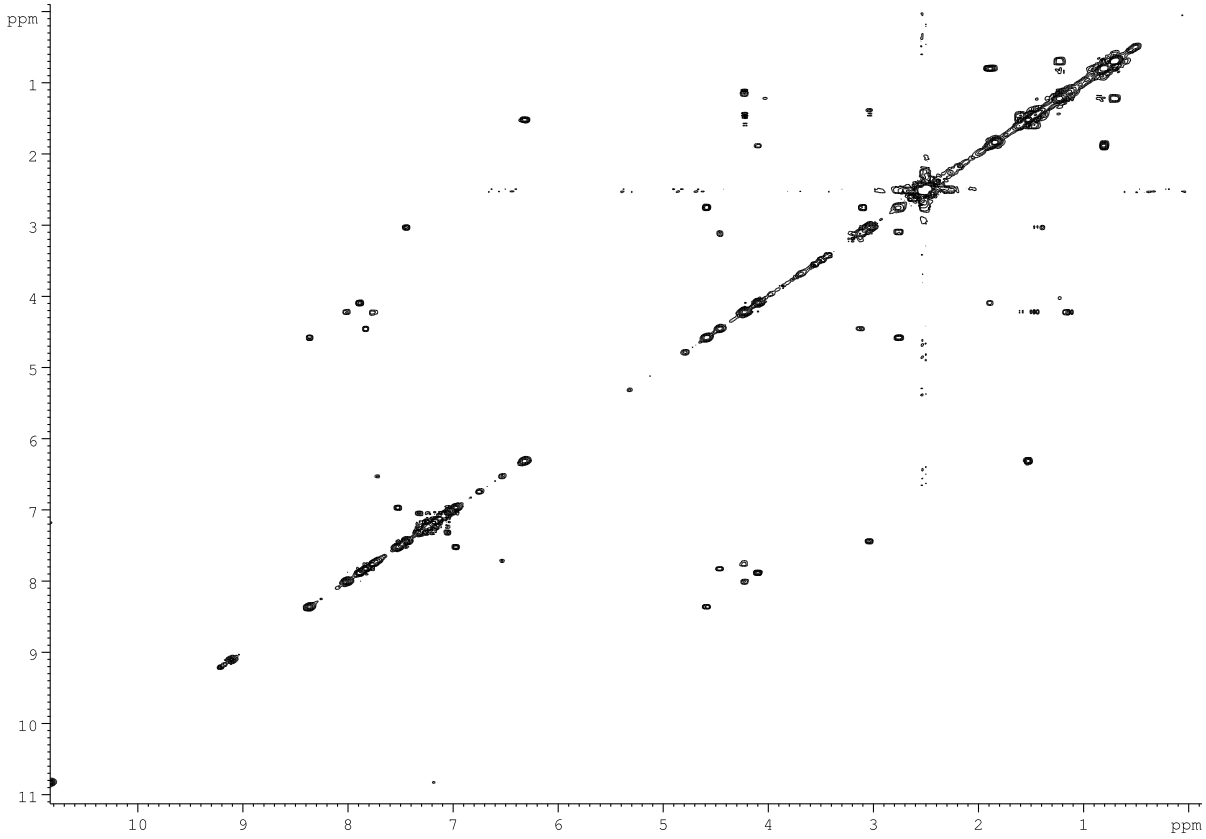


Figure S16. ^{1}H, ^{1}H COSY spectrum of **3** in DMSO-d_6

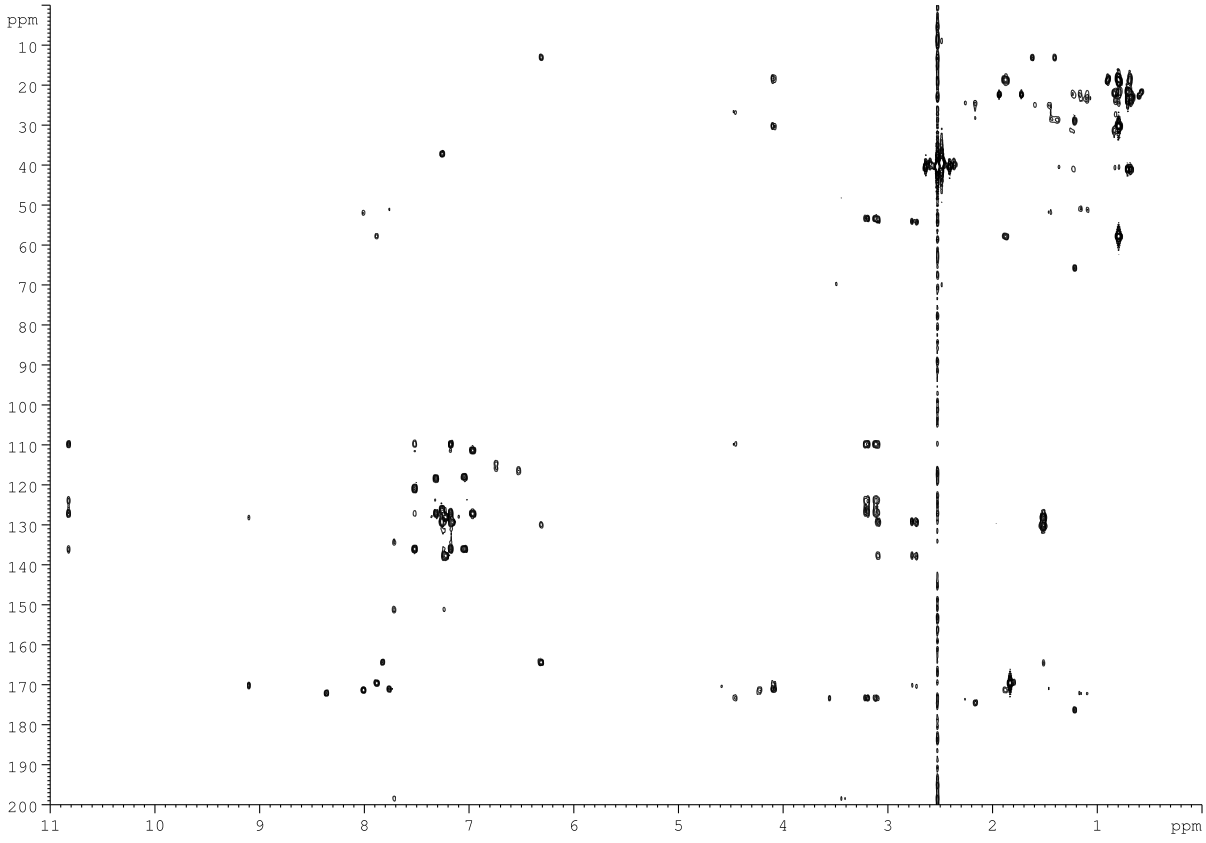


Figure S17. ^{1}H, ^{13}C HMBC spectrum of **3** in DMSO-d_6

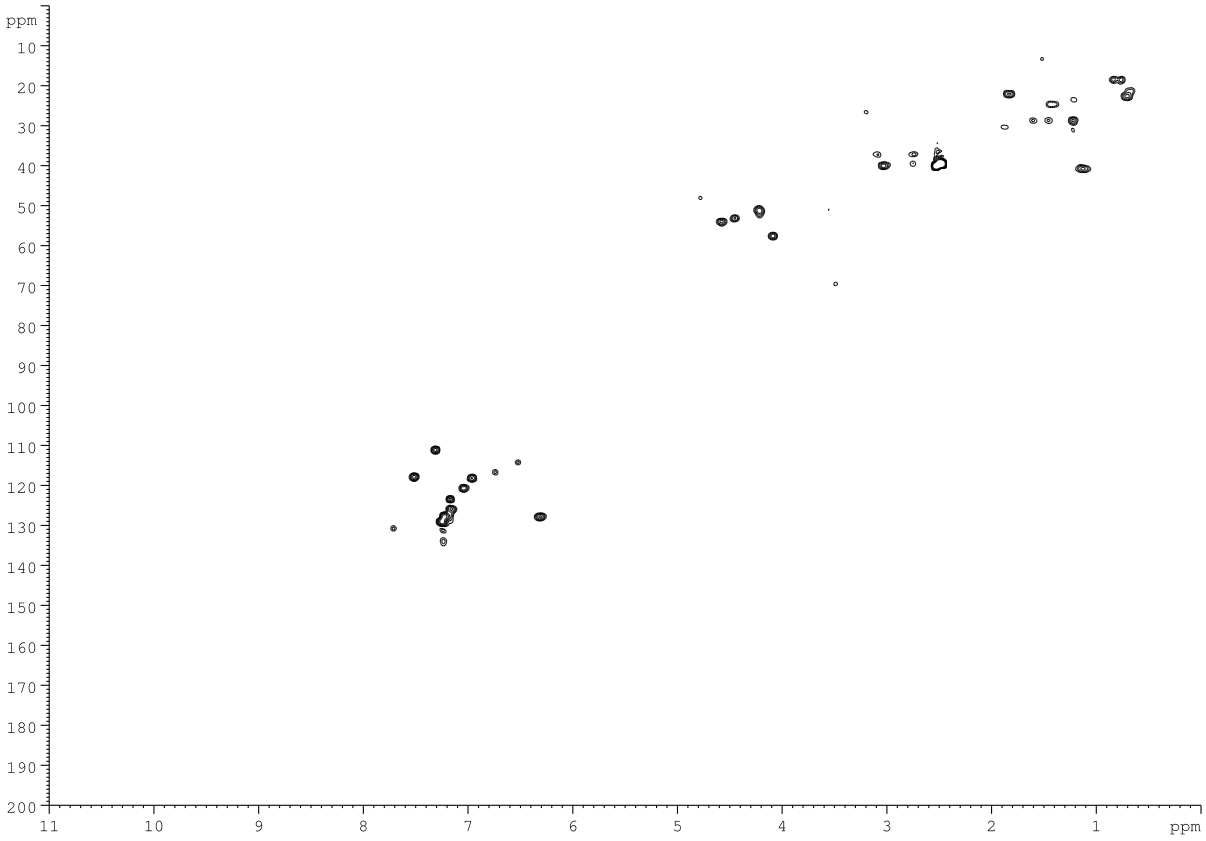


Figure S18. ^{1}H, ^{13}C HSQC spectrum of **3** in DMSO-d_6

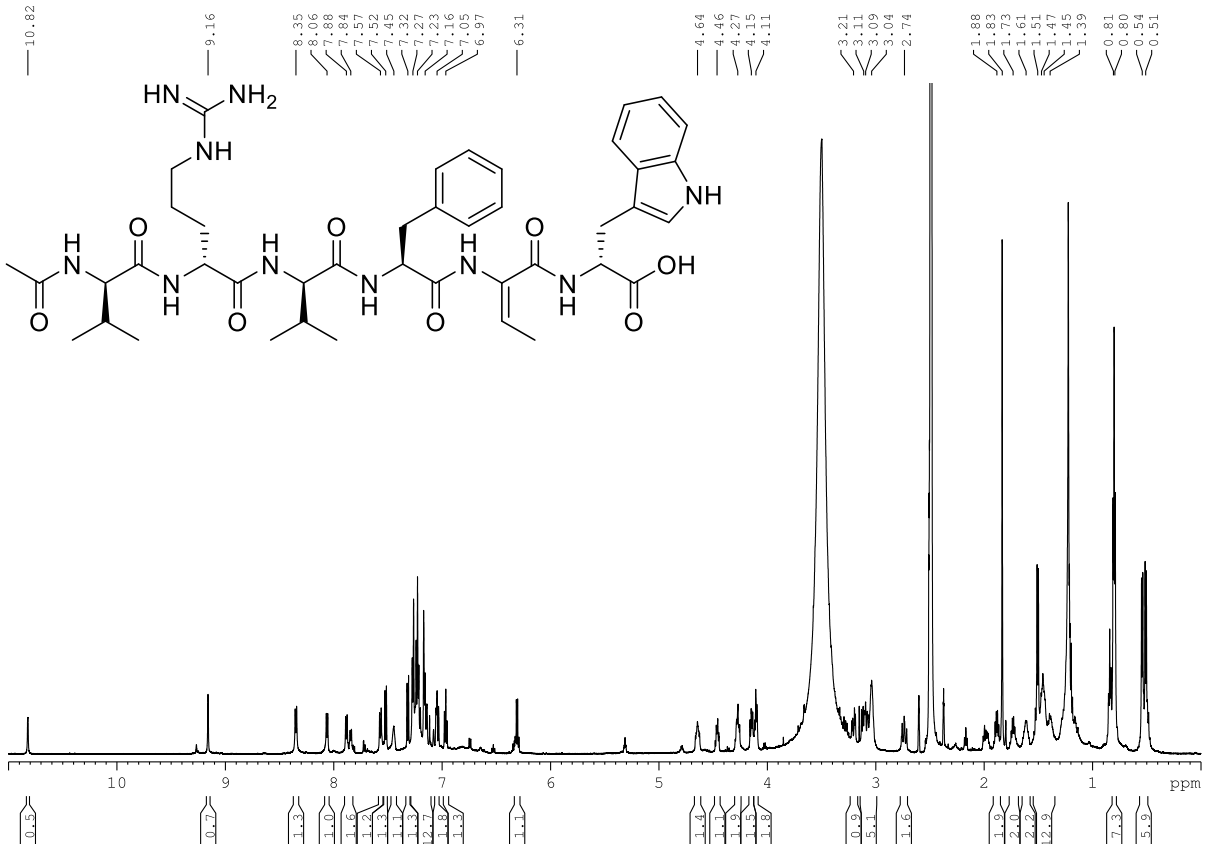


Figure S19. ^{1}H NMR spectrum of **4** in DMSO-d_6

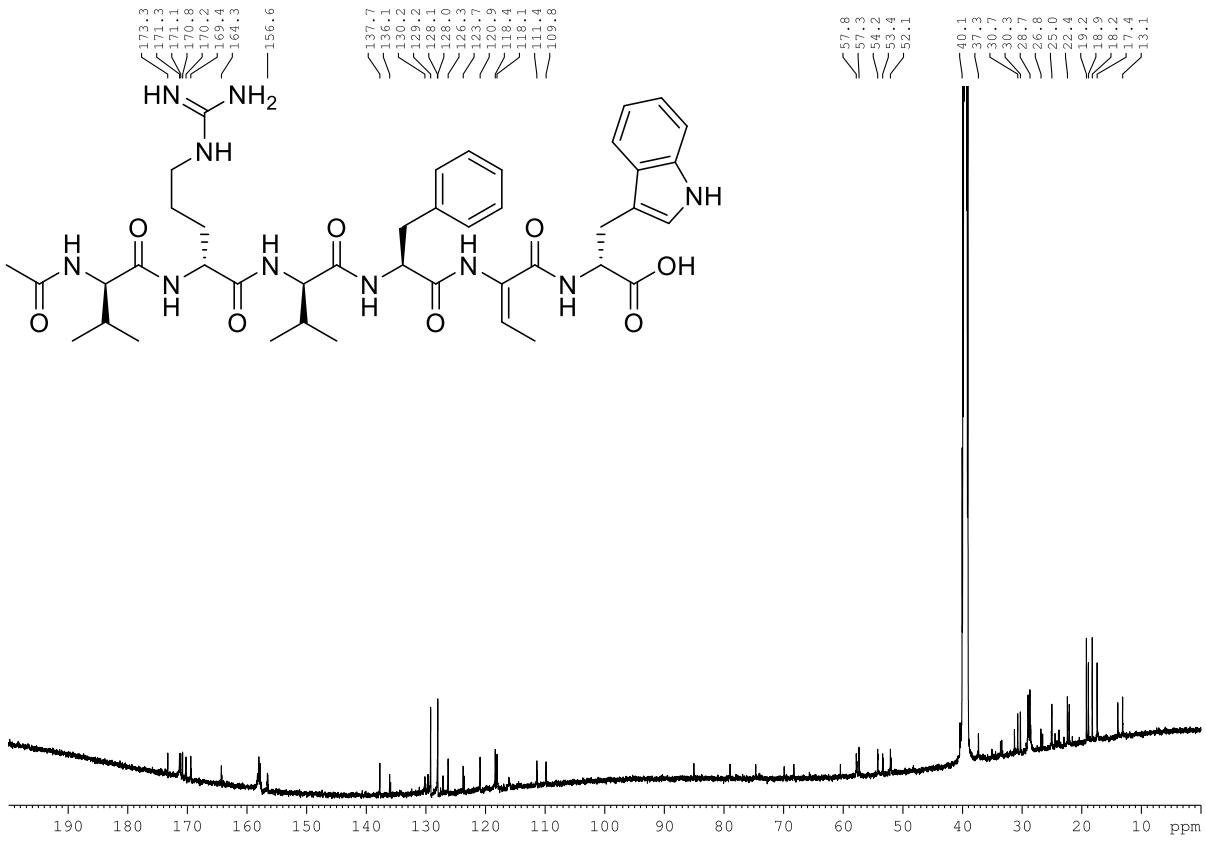


Figure S20. ^{1}H decoupled ^{13}C NMR spectrum of **4** in DMSO-d_6

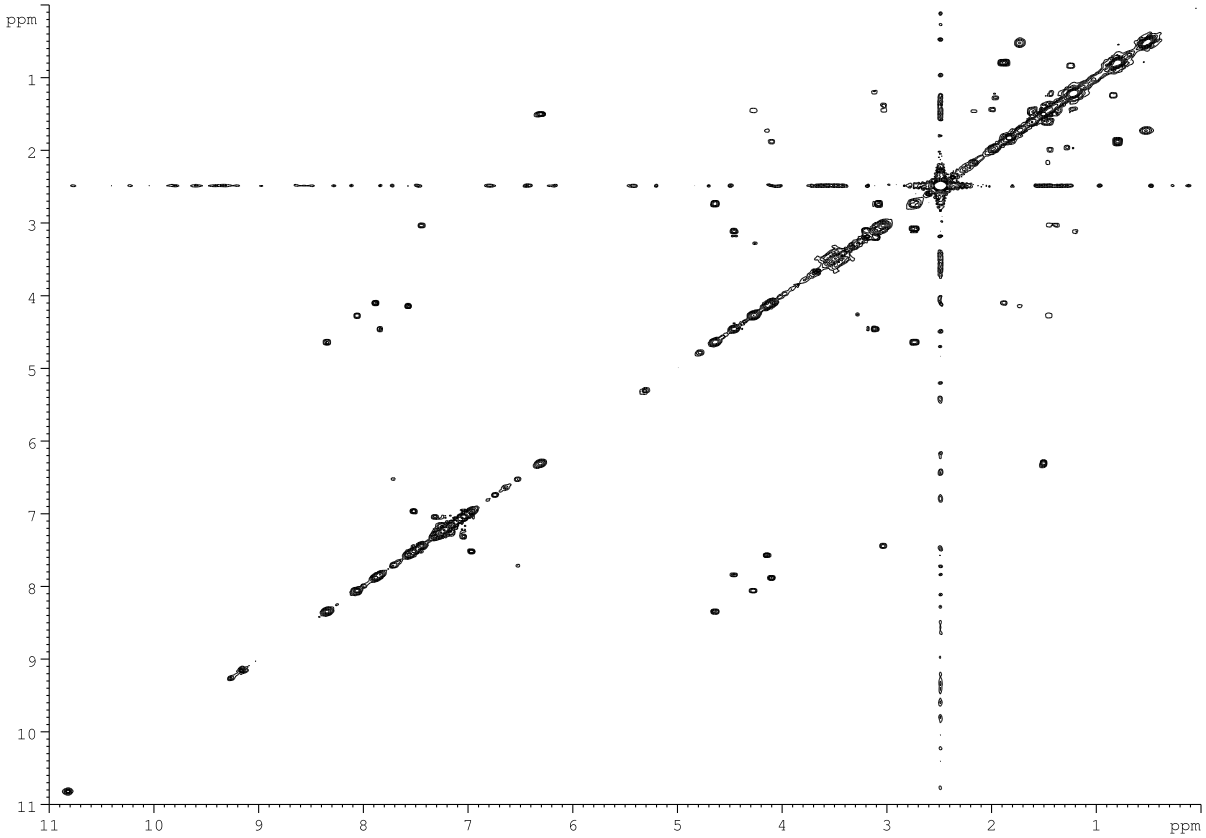


Figure S21. ^{1}H, ^{1}H COSY spectrum of **4** in DMSO-d_6

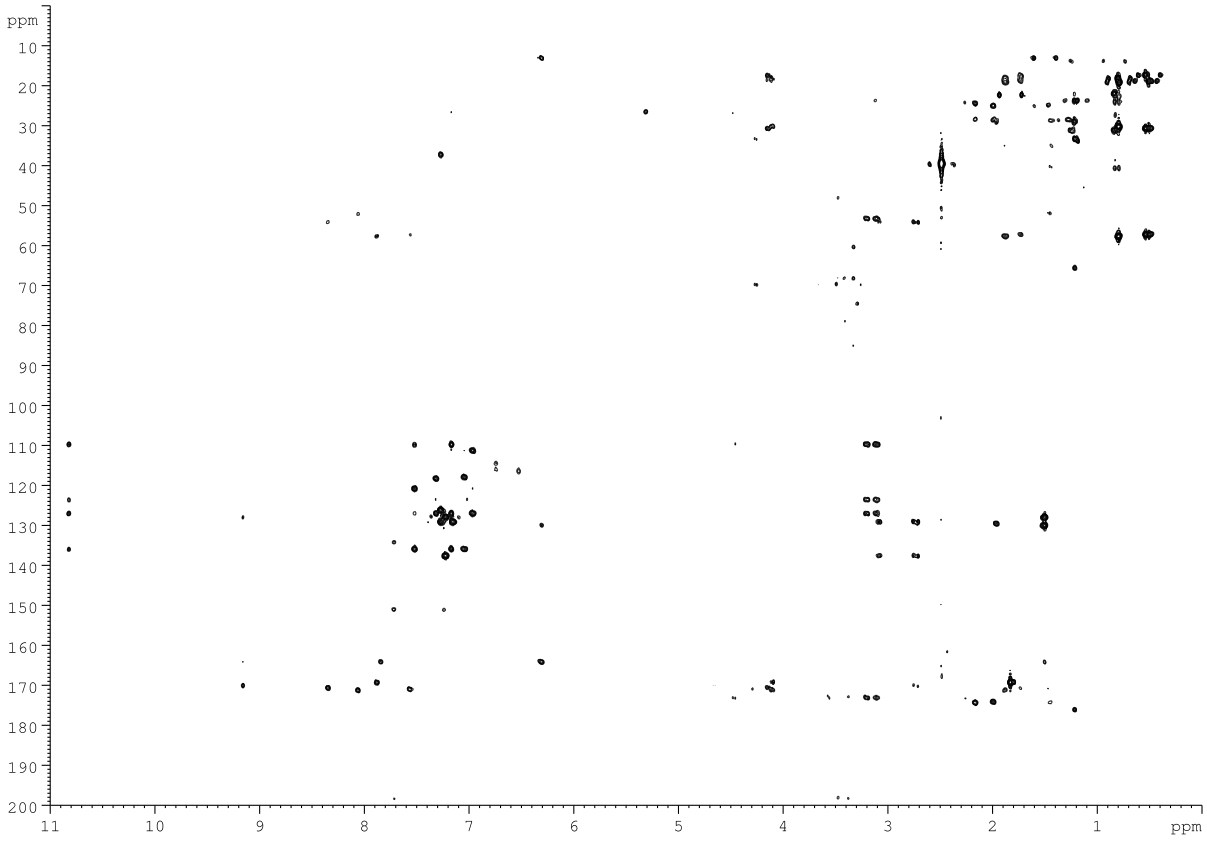


Figure S22. ^{1}H, ^{13}C HMBC spectrum of **4** in DMSO-d_6

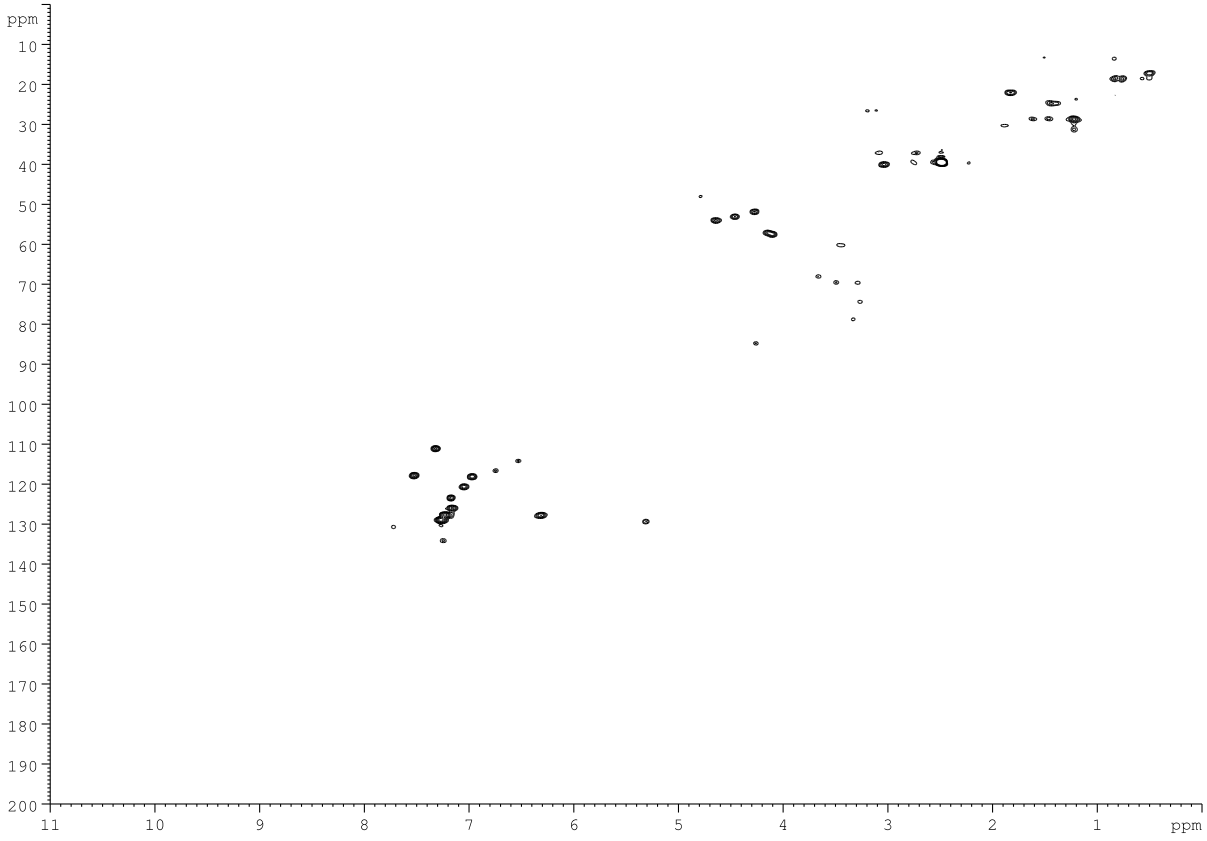


Figure S23. ^{1}H, ^{13}C HSQC spectrum of **4** in DMSO-d_6

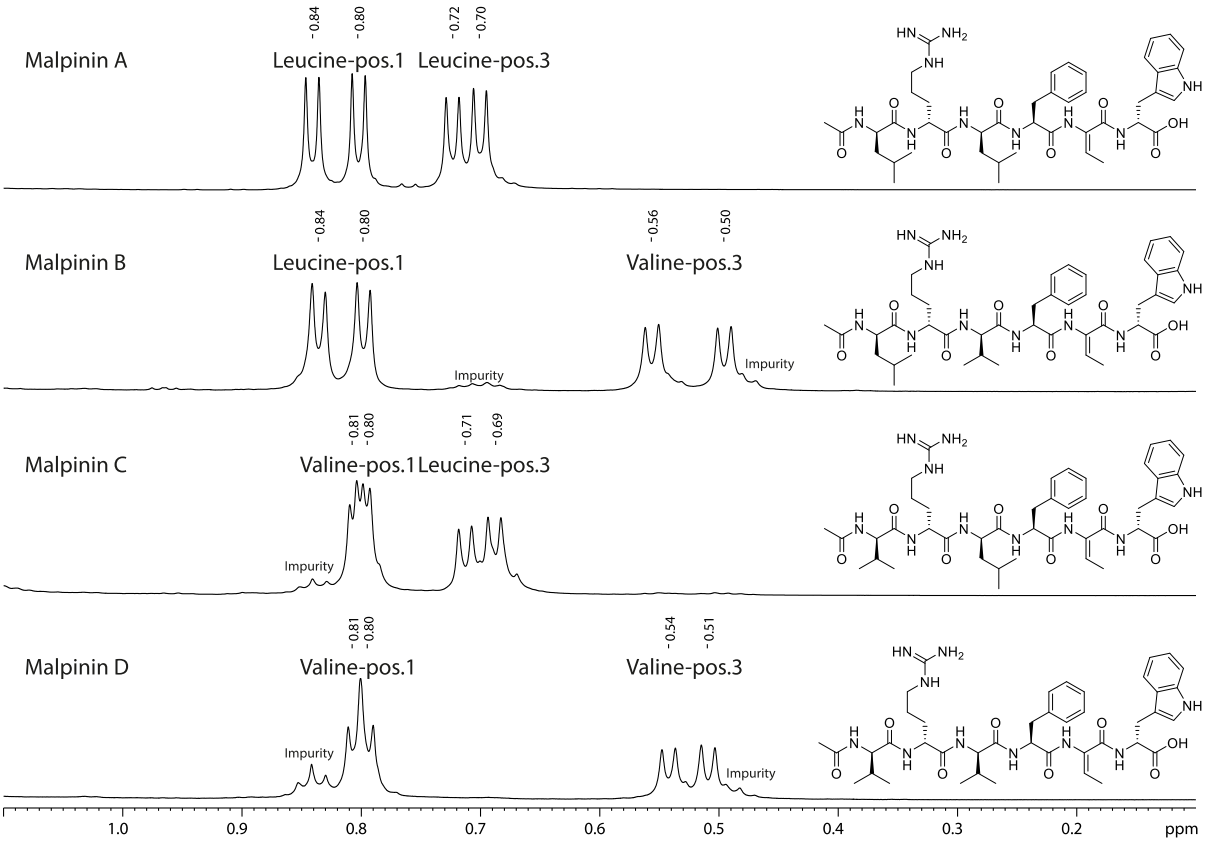


Figure S24. Upfield region of the ^{1}H NMR spectra of **1** - **4**. The chemical shift signals of the methyl moieties of valine and leucine clearly demonstrate the amino acid exchange on position 1 (δ_H(leu/val) = 0.85 - 0.75 ppm), and 3 (δ_H(leu) = 0.72 - 0.67 ppm or δ_H(val) = 0.55 - 0.49 ppm).

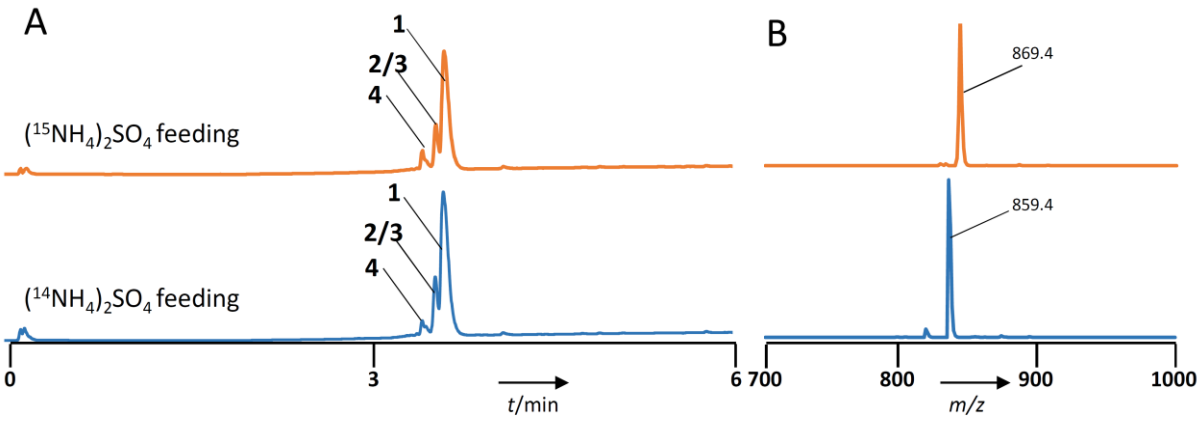


Figure S25. ^{15}N stable isotope labeling experiment. AMM cultures of *M. alpina* ATCC32222 were supplemented with 35 mM $(^{15}NH_4)_2SO_4$ (upper lane) or 35 mM $(^{14}NH_4)_2SO_4$ (lower lane, control) and incubated for 12 days at 25°C,180 RPM. Mycelium was extracted and subjected to UHPLC-MS analysis. ESI-MS was carried out in positive ionization mode $[M + H]^+$. (**A**) UHPLC profile of butanolic extracts. (**B**) Mass spectrum $[M + H]^+$ of **1** at t_R = 3.8 min. The mass shift of 10 compared to the control indicates the incorporation of 10 nitrogen atoms in **1**, supporting the presence of a guanidine group in **1.**

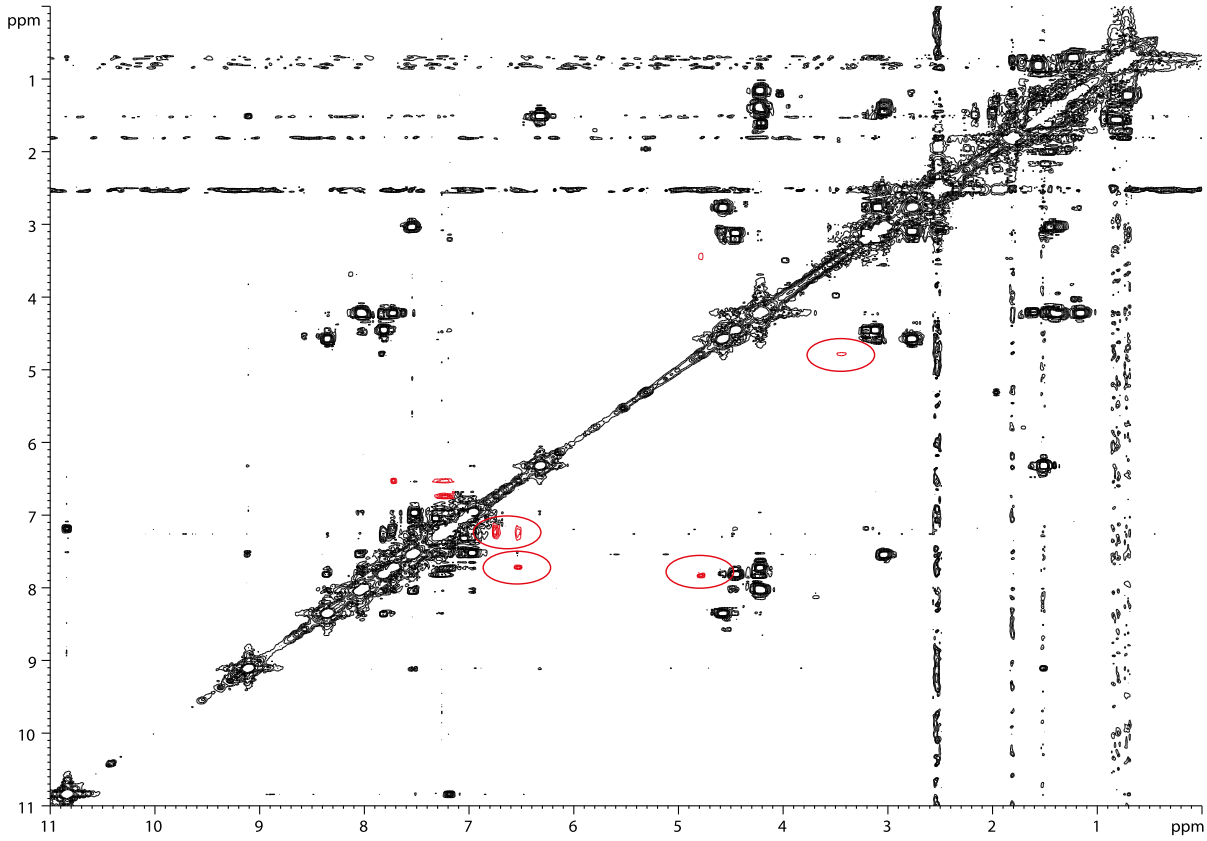


Figure S26. ^{1}H, ^{1}H COSY spectrum of **6** in DMSO-d_6

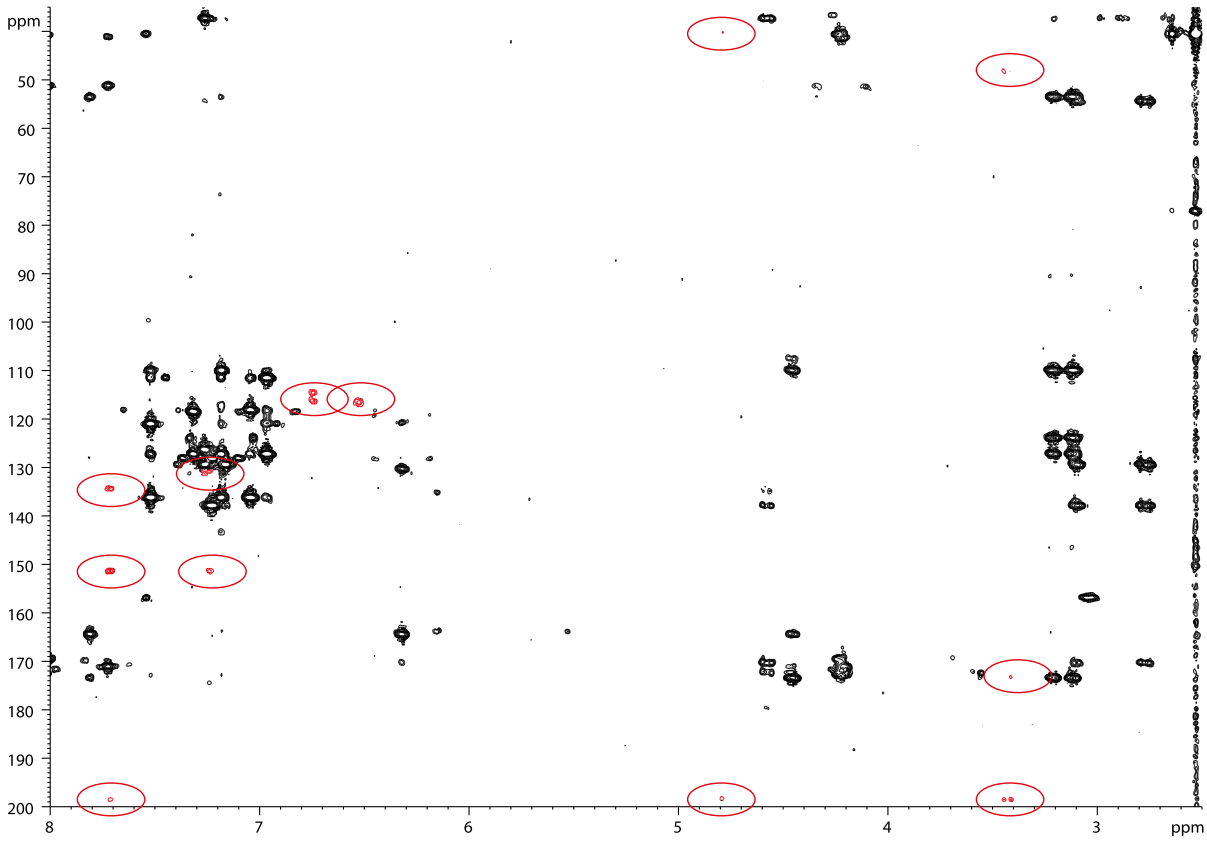


Figure S27. ^{1}H, ^{13}C HMBC spectrum of **6** in DMSO-d_6

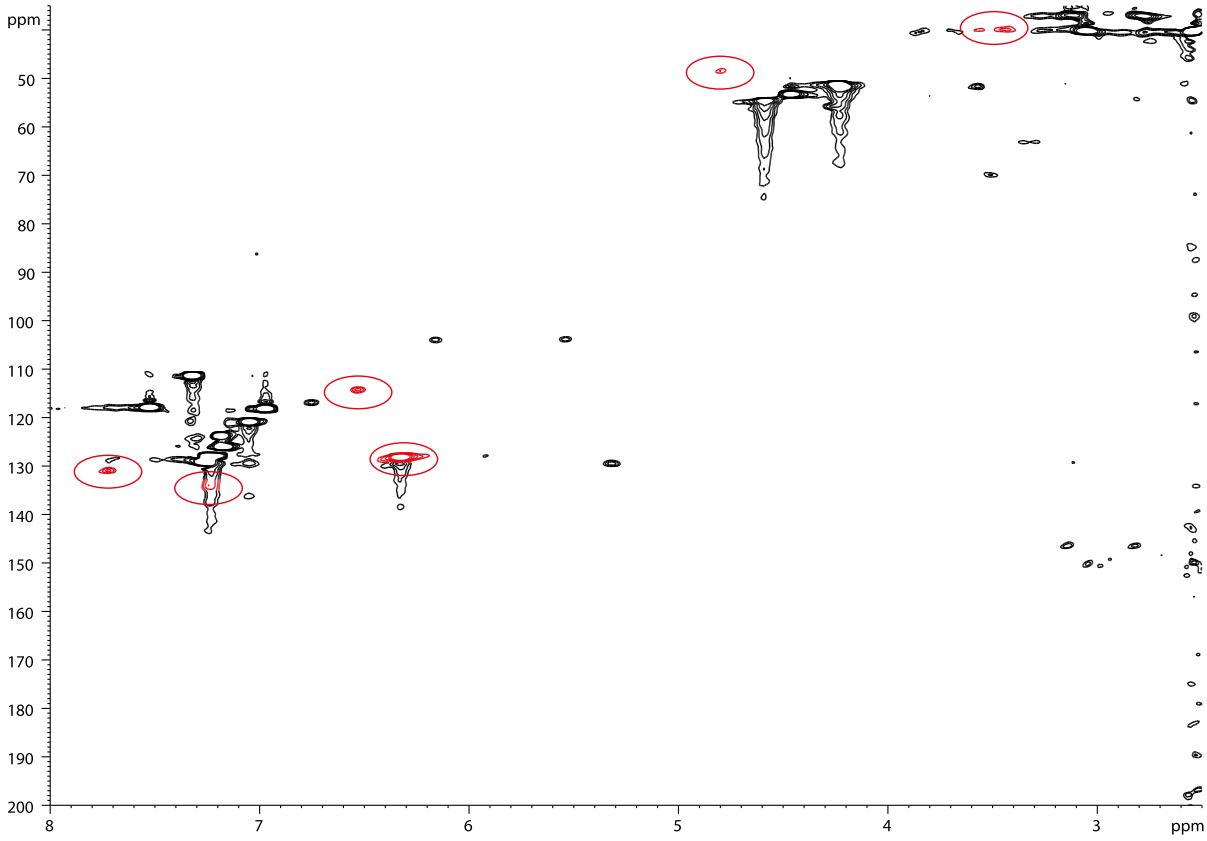


Figure S28. ^{1}H, ^{13}C HSQC spectrum of **6** in DMSO-d_6

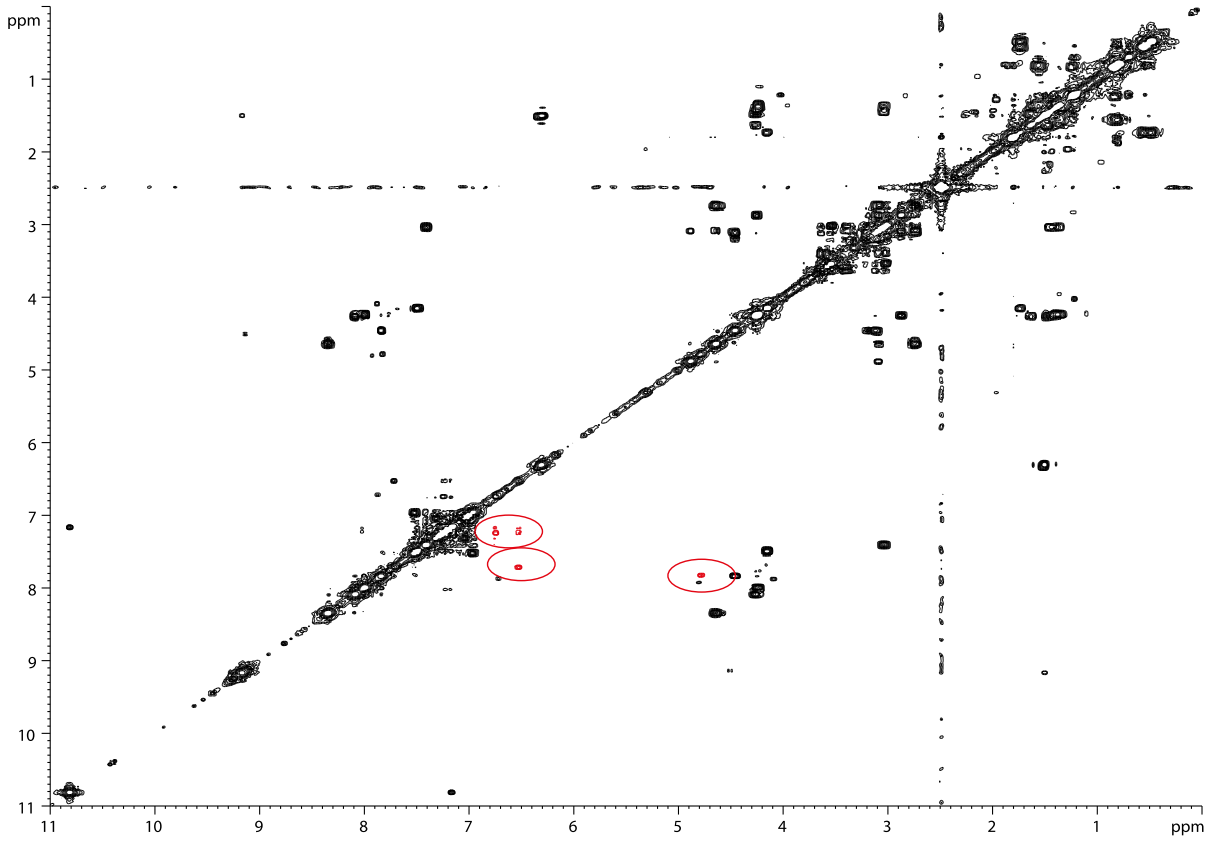


Figure S29. ^{1}H, ^{1}H COSY spectrum of **7** in DMSO-d_6

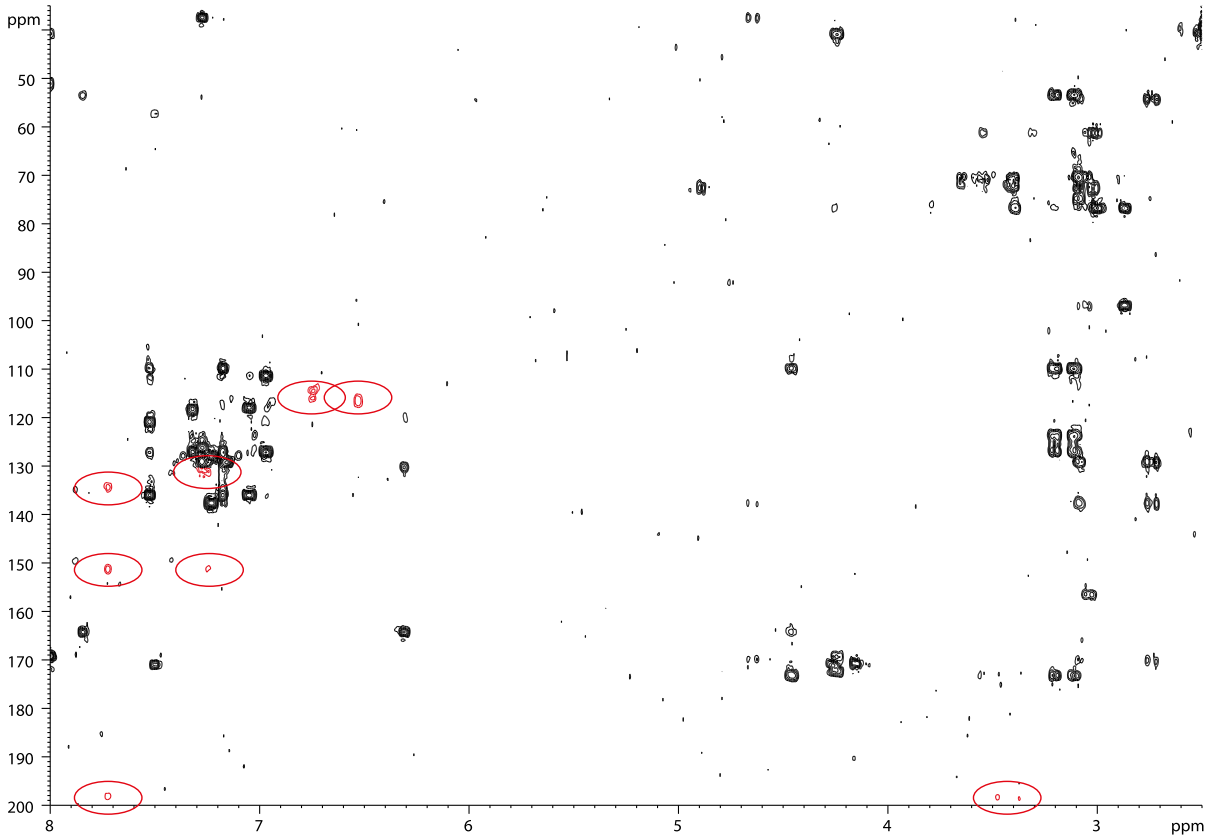


Figure S30. ^{1}H, ^{13}C HMBC spectrum of **7** in DMSO-d_6

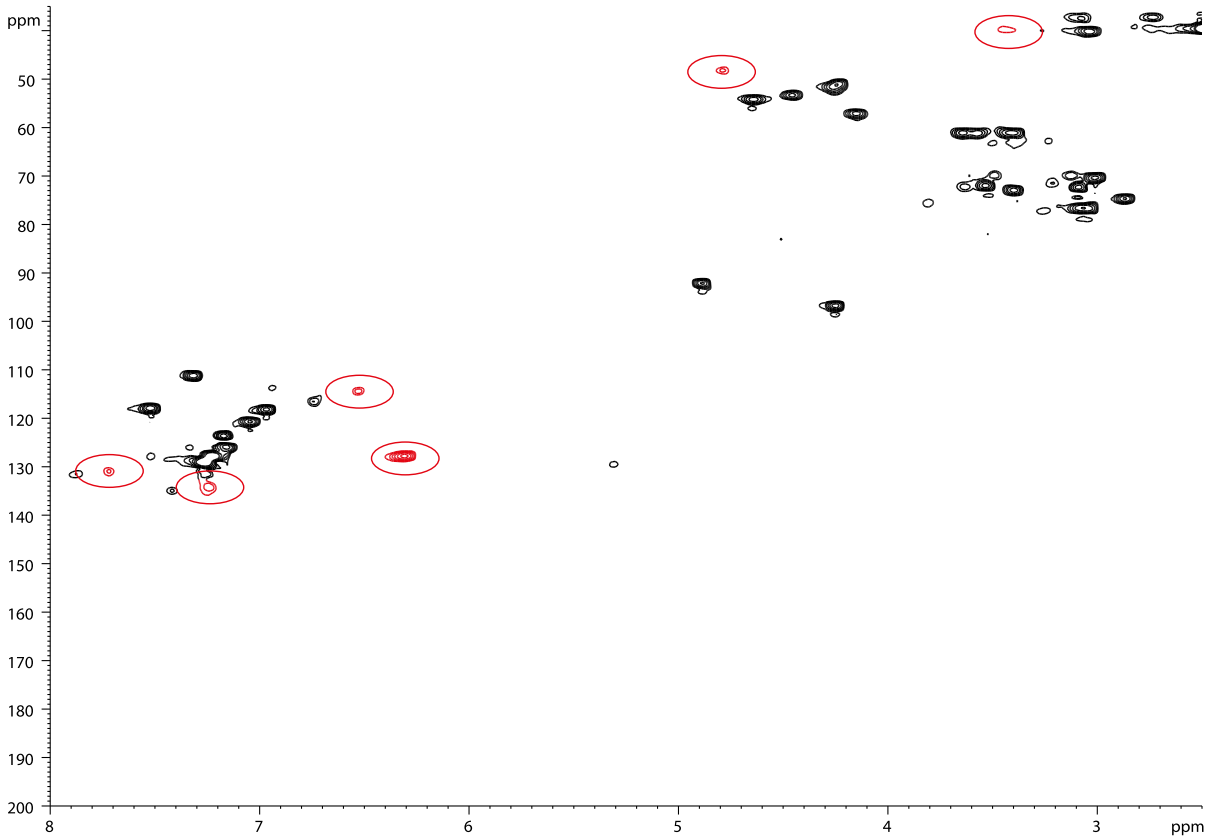


Figure S31. ^{1}H, ^{13}C HSQC spectrum of **7** in DMSO-d_6

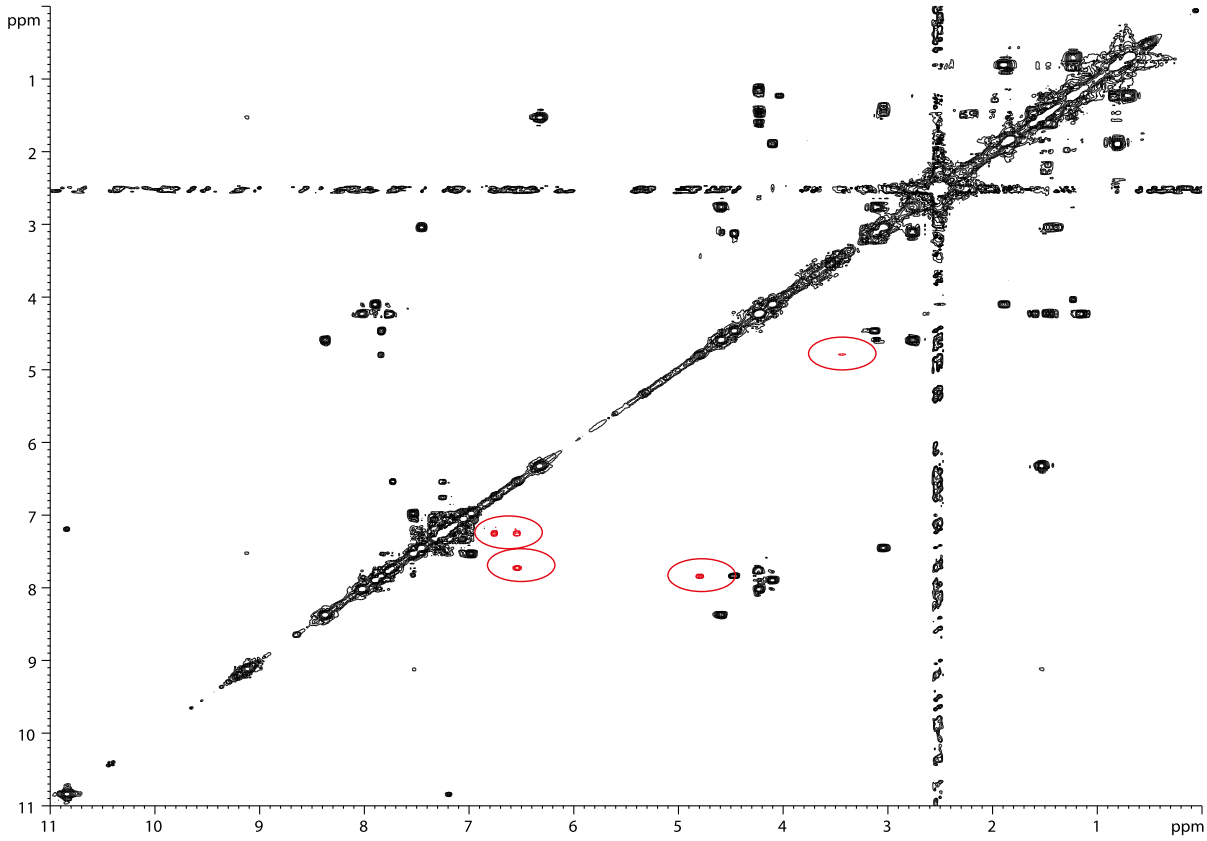


Figure S32. ^{1}H, ^{1}H COSY spectrum of **8** in DMSO-*d6*

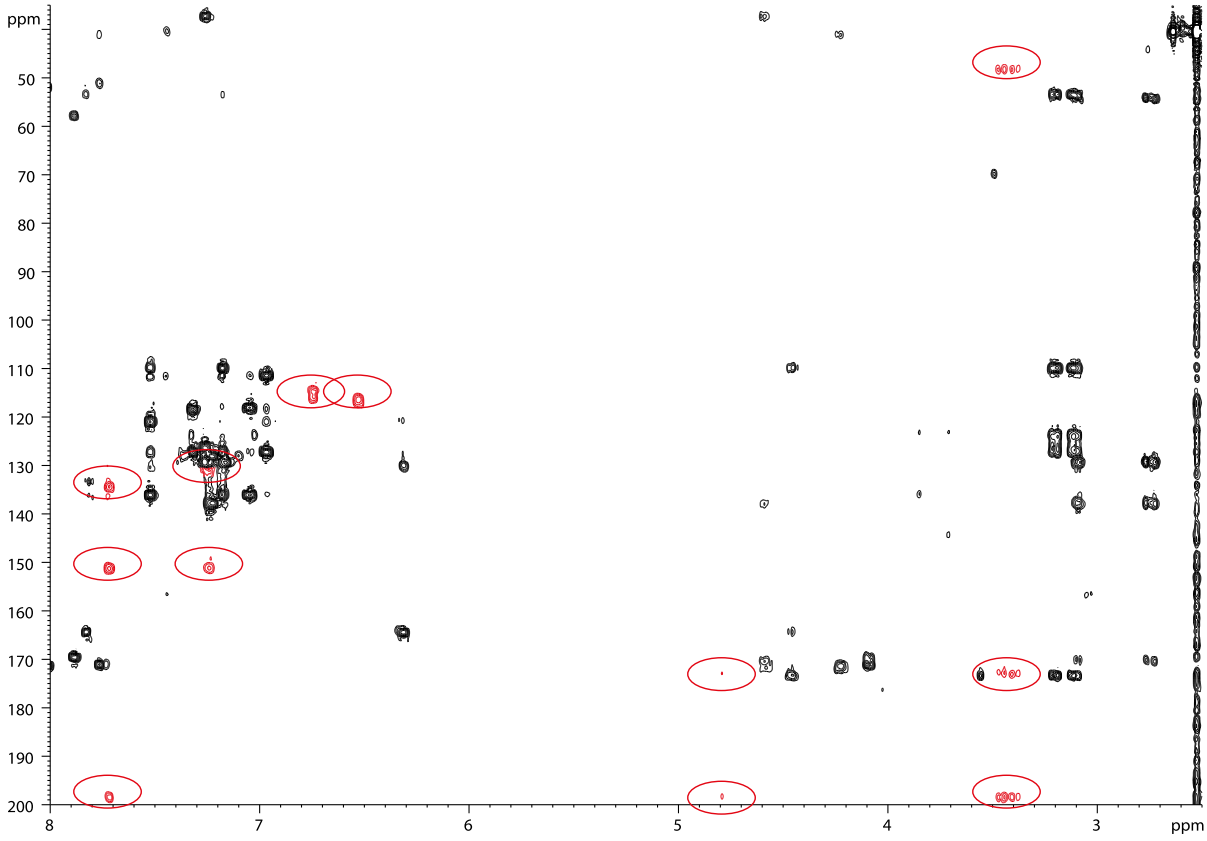


Figure S33. ^{1}H, ^{13}C HMBC spectrum of **8** in DMSO-d_6

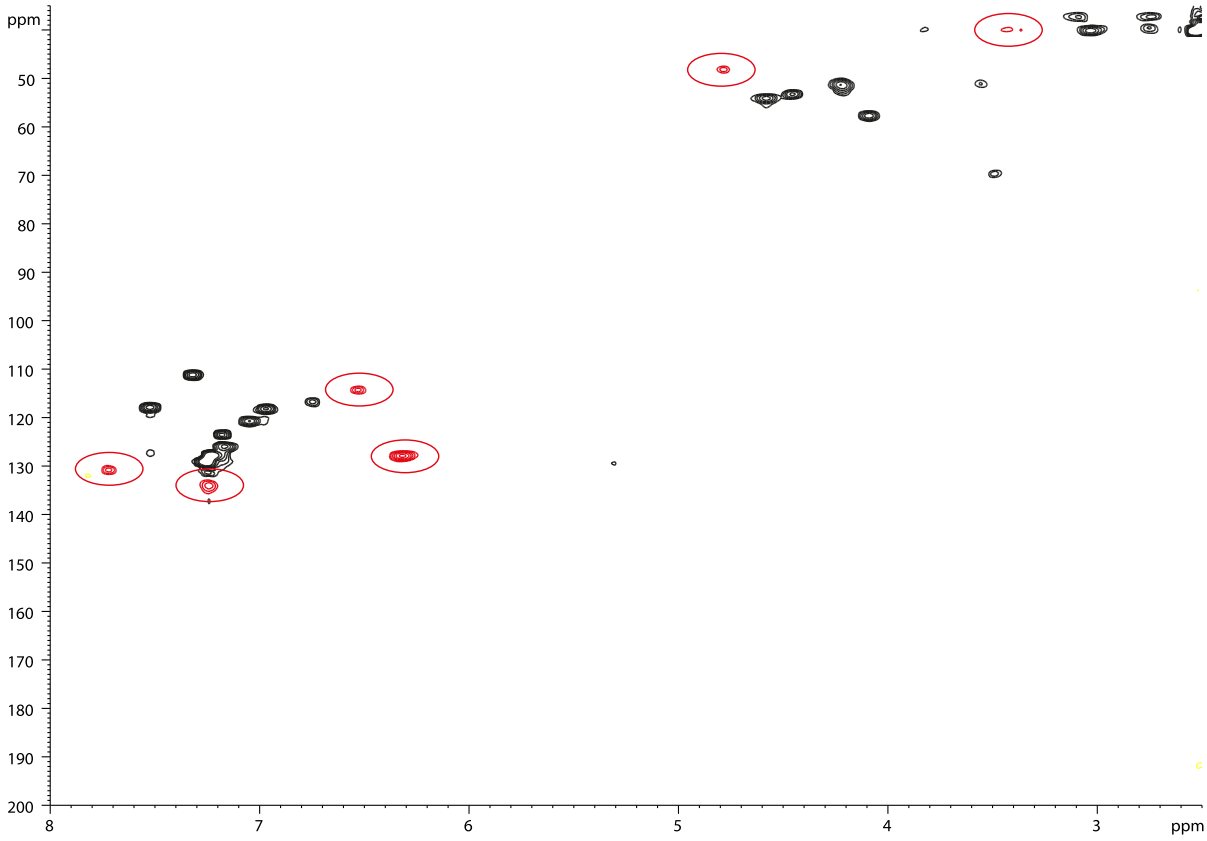


Figure S34. ^{1}H, ^{13}C HSQC spectrum of **8** in DMSO-d_6

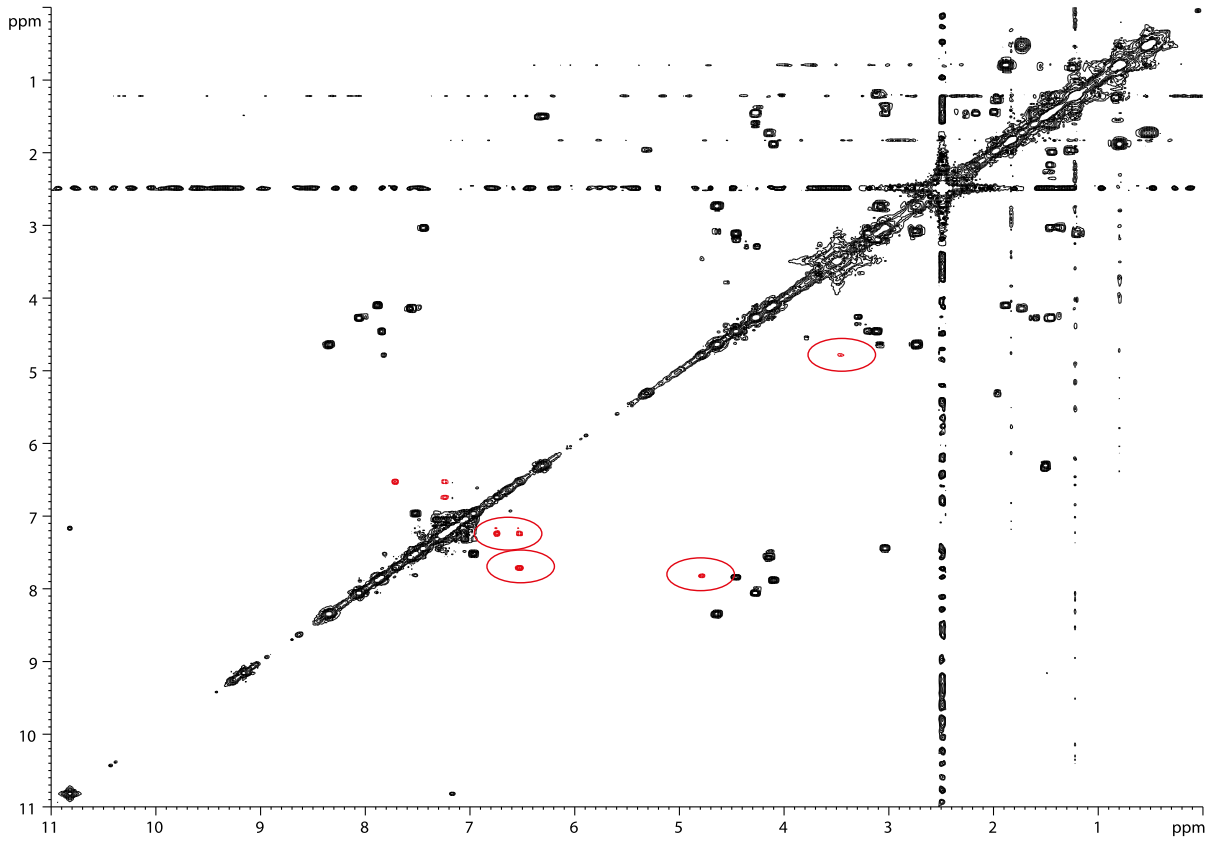


Figure S35. ^{1}H, ^{1}H COSY spectrum of **9** in DMSO-d_6

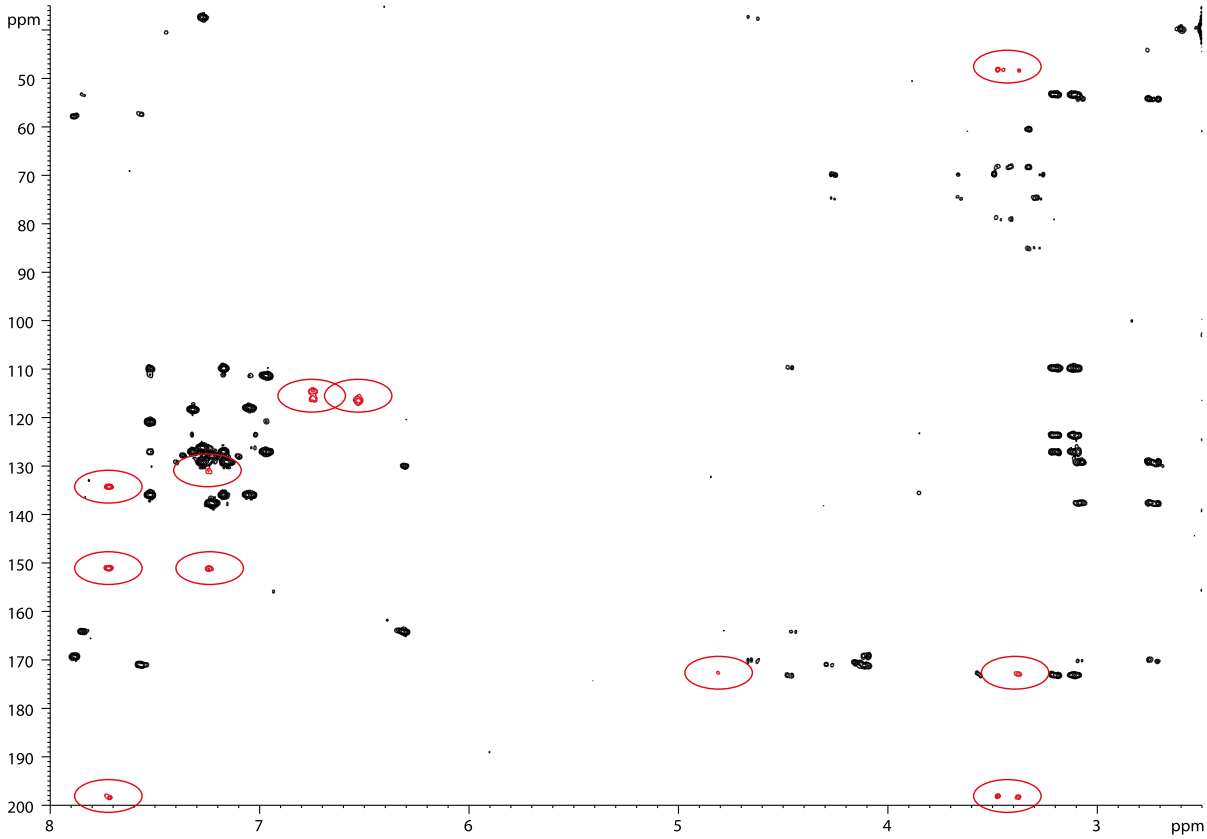


Figure S36. ^{1}H, ^{13}C HMBC spectrum of **9** in DMSO-d_6

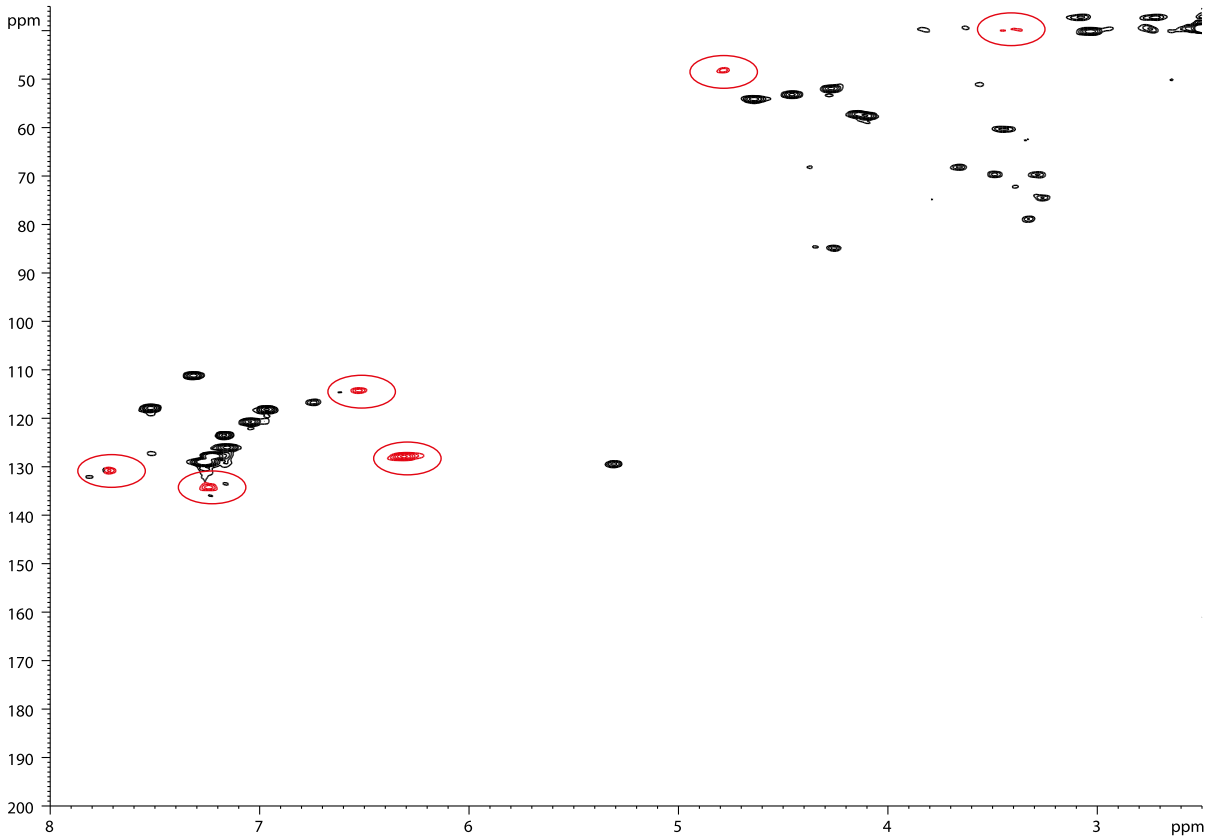


Figure S37. ^{1}H, ^{13}C HSQC spectrum of **9** in DMSO-d_6

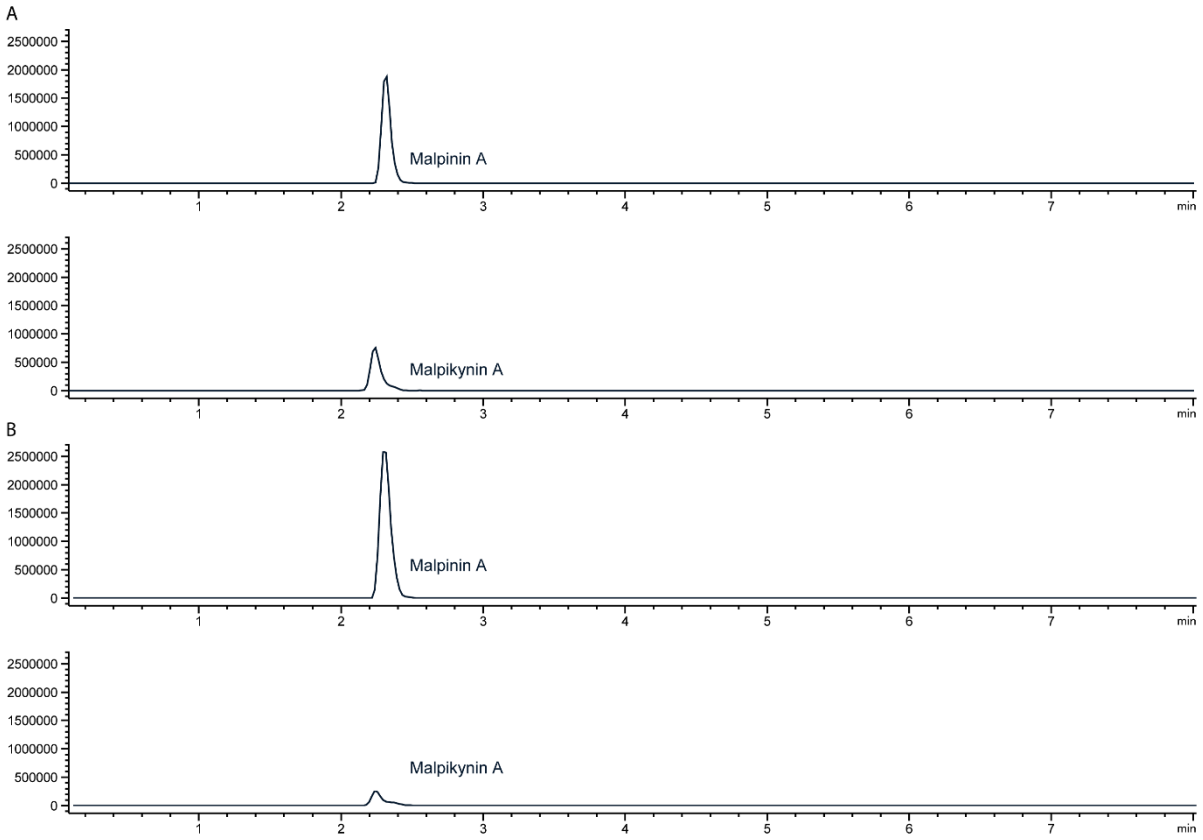


Figure S38. Oxidative conversion of malpinin A (**1**) to malpikynin (**6**)**.** 10 µL of H_2O_2 or H_2O (control) was added to 90 µL of **1** in methanol and analyzed after 7 days of incubation at room temperature via UHPLC-MS. **A.** Relative abundance of **1** and **6** after treatment with H_2O_2. **B.** Relative abundance of **1** and **6** after treatment with H_2O (control). While the quantity of **6** increased, the amount of **1** decreased under H_2O_2-treatment.

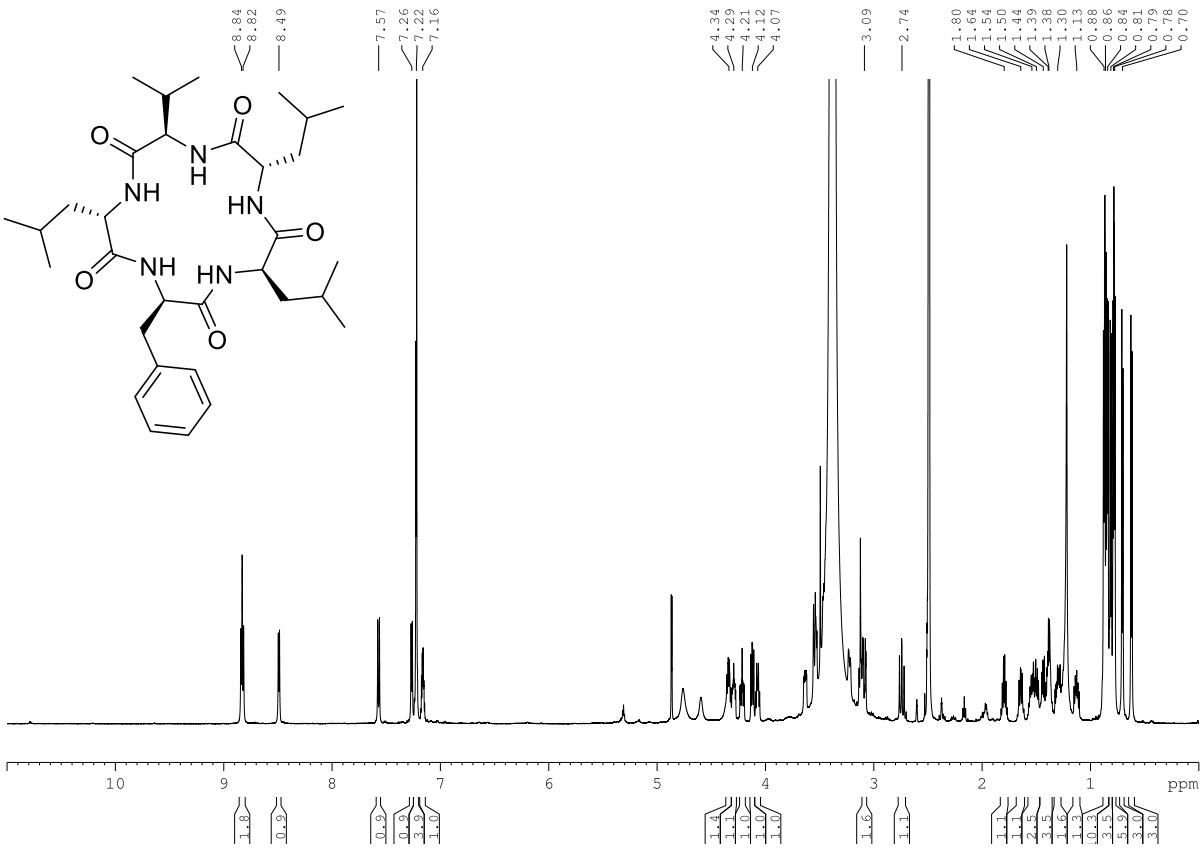


Figure S39. [1]H NMR- spectrum of **11** in DMSO-d_6

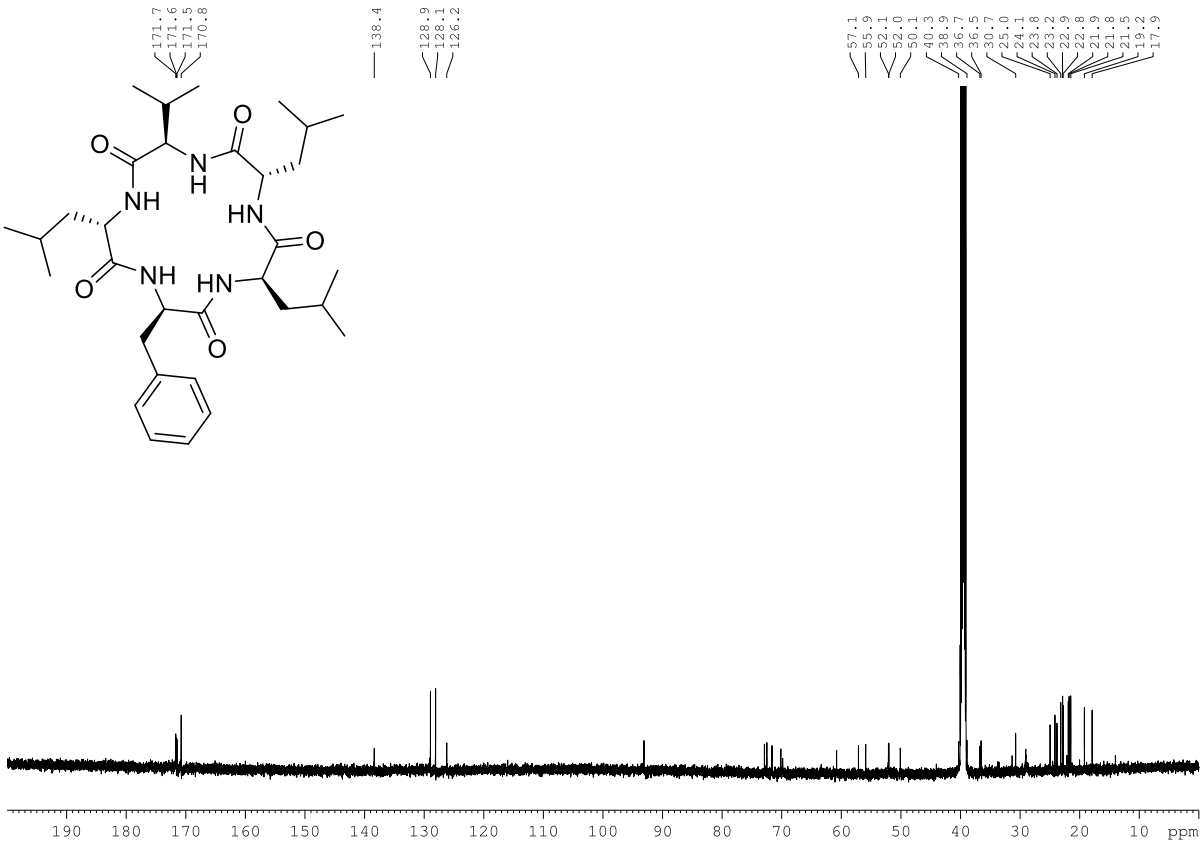


Figure S40. [1]H decoupled [13]C NMR spectrum of **11** in DMSO-d_6

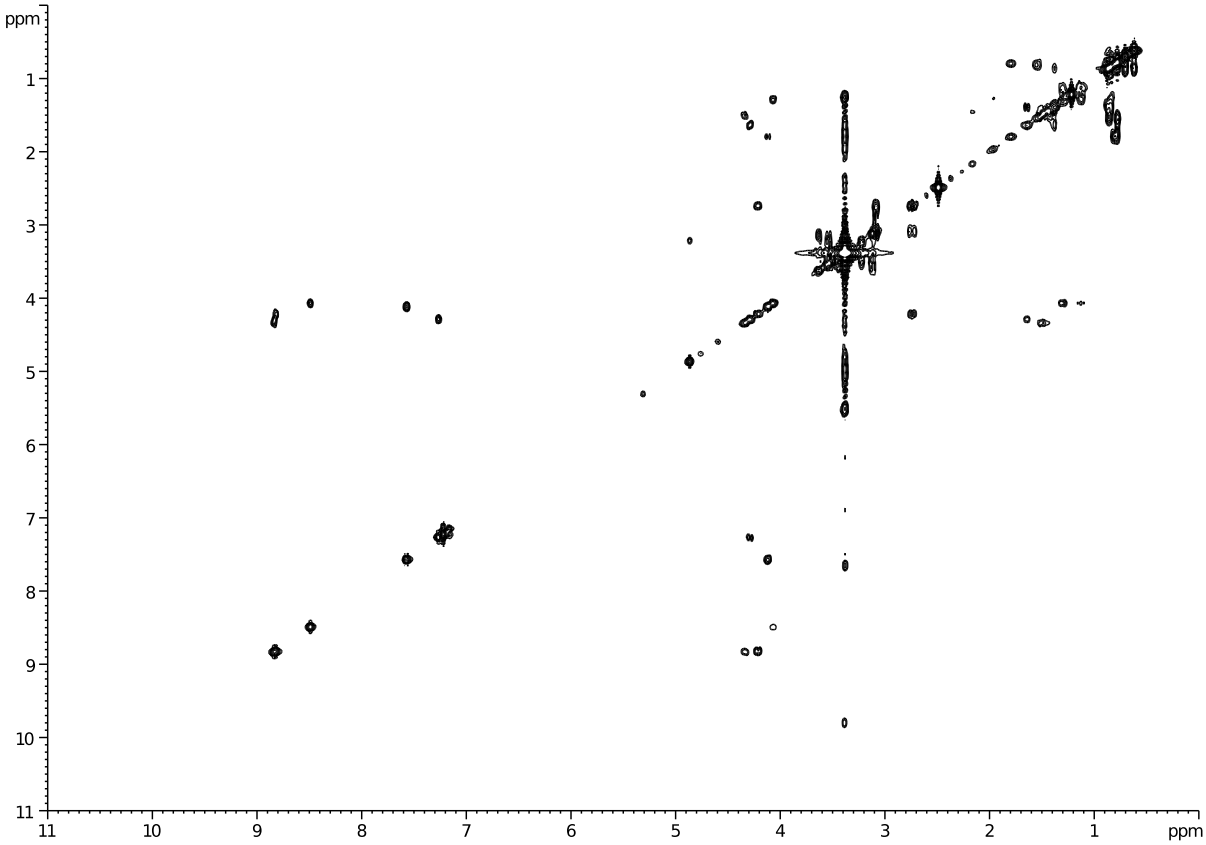


Figure S41. ^{1}H, ^{1}H COSY spectrum of **11** in DMSO-d_6

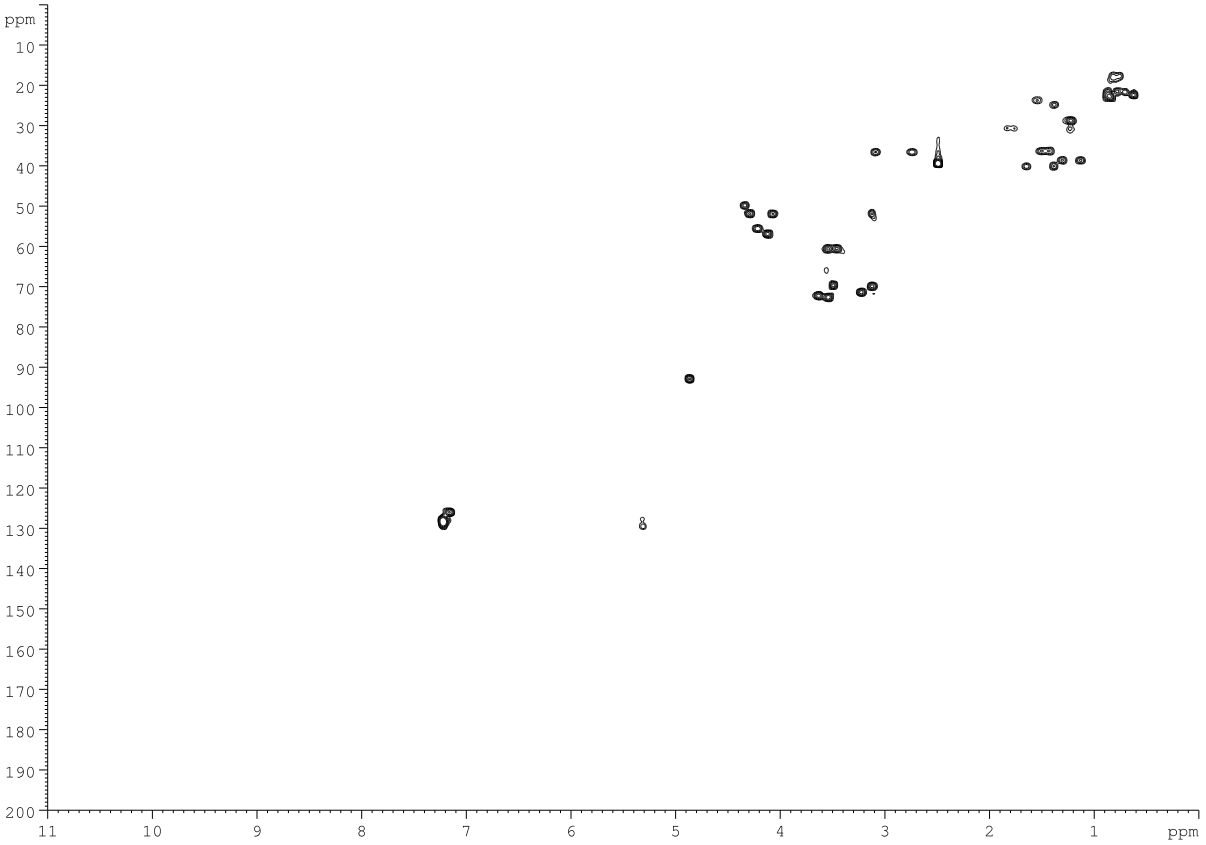


Figure S42. ^{1}H, ^{13}C HSQC spectrum of **11** in DMSO-d_6

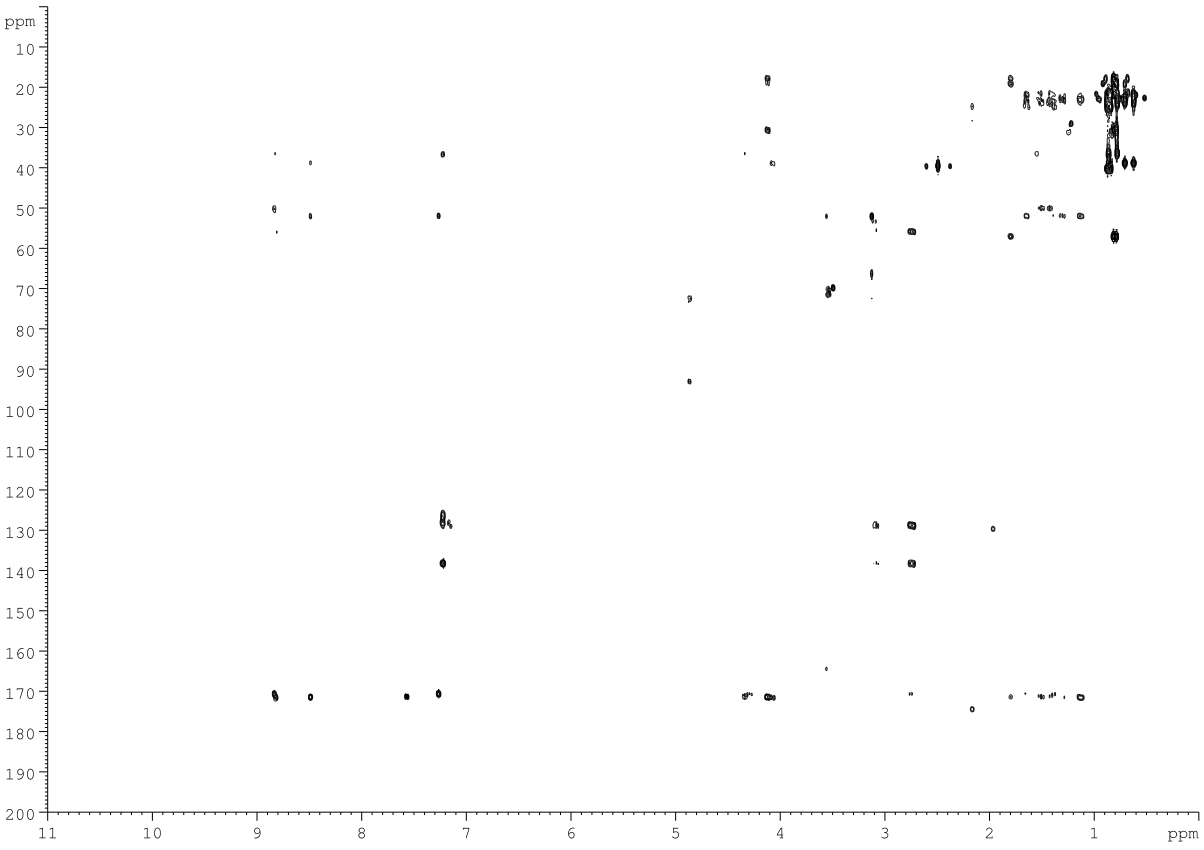


Figure S43. ^{1}H, ^{13}C HMBC spectrum of **11** in DMSO-d_6

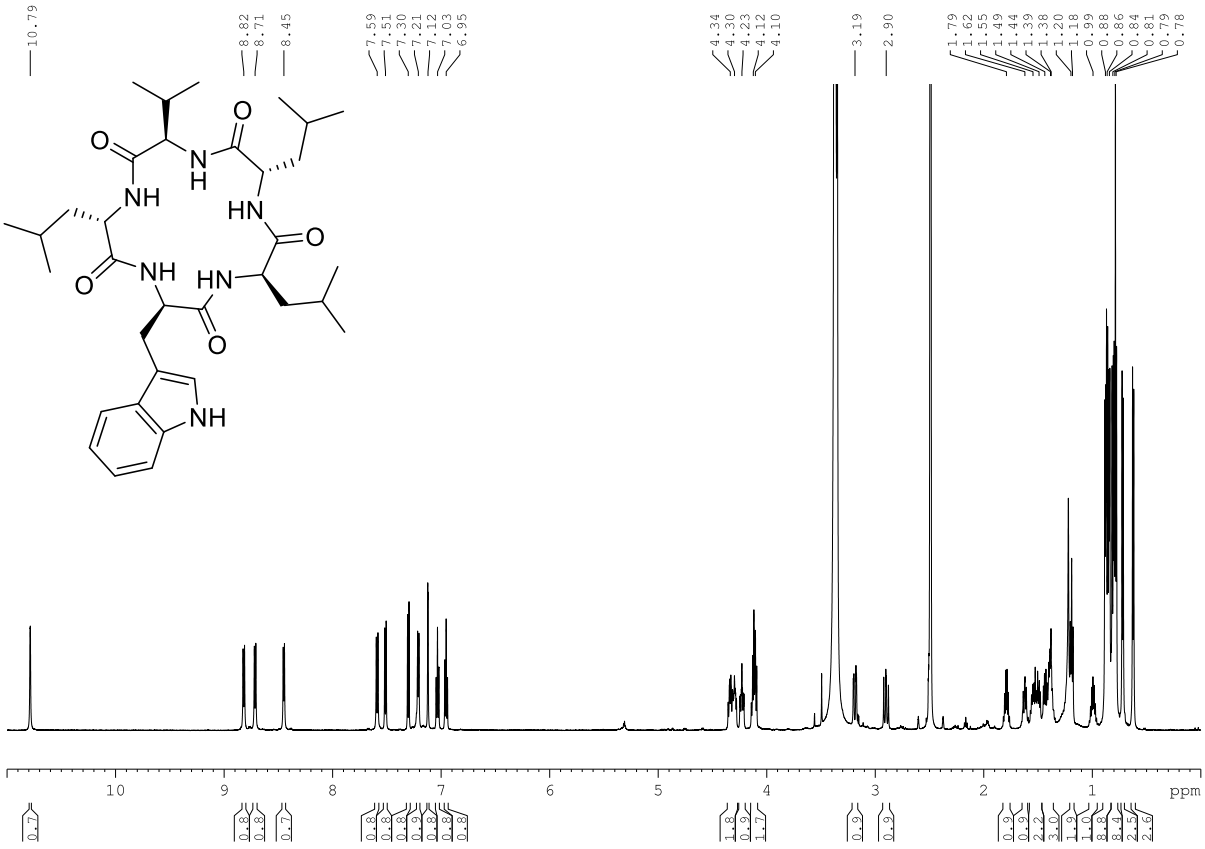


Figure S44. ^{1}H NMR- spectrum of **12** in DMSO-d_6

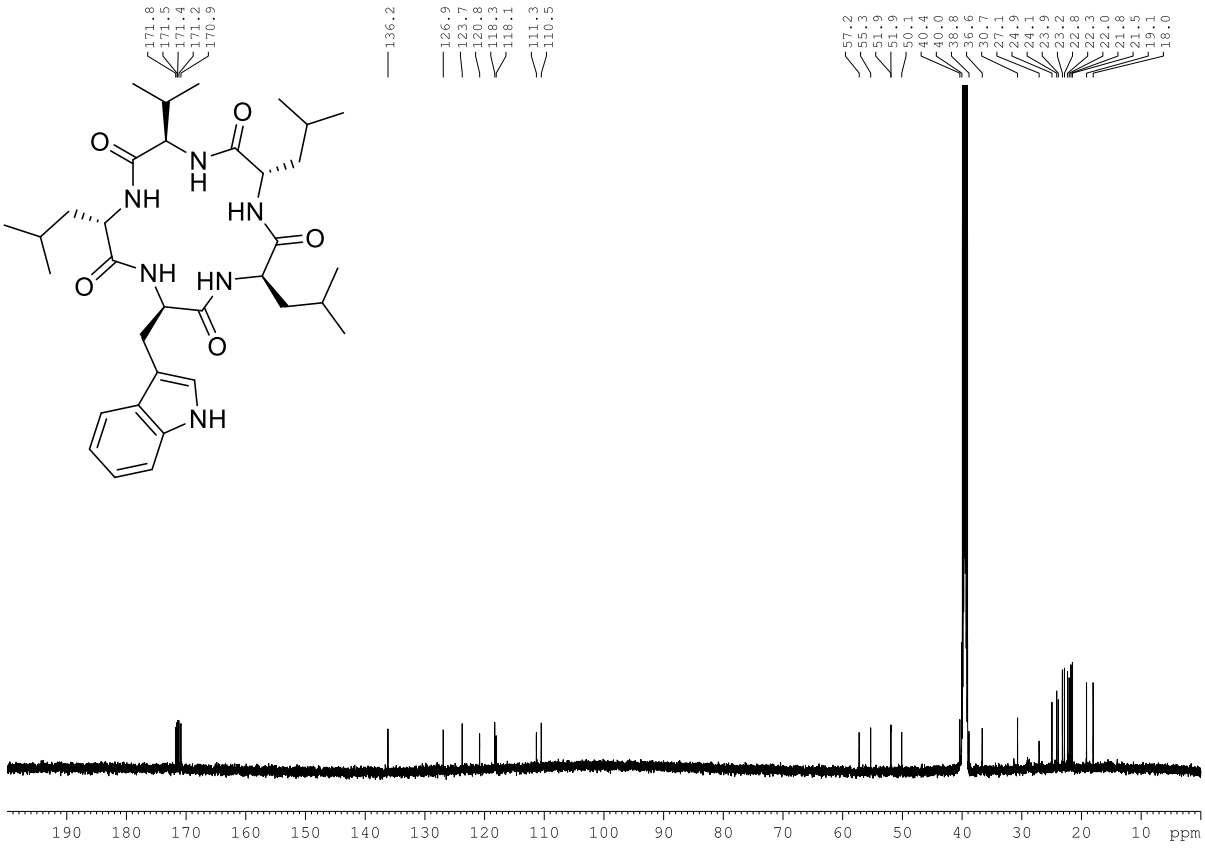


Figure S45. ^{1}H decoupled ^{13}C NMR spectrum of **12** in DMSO-d_6

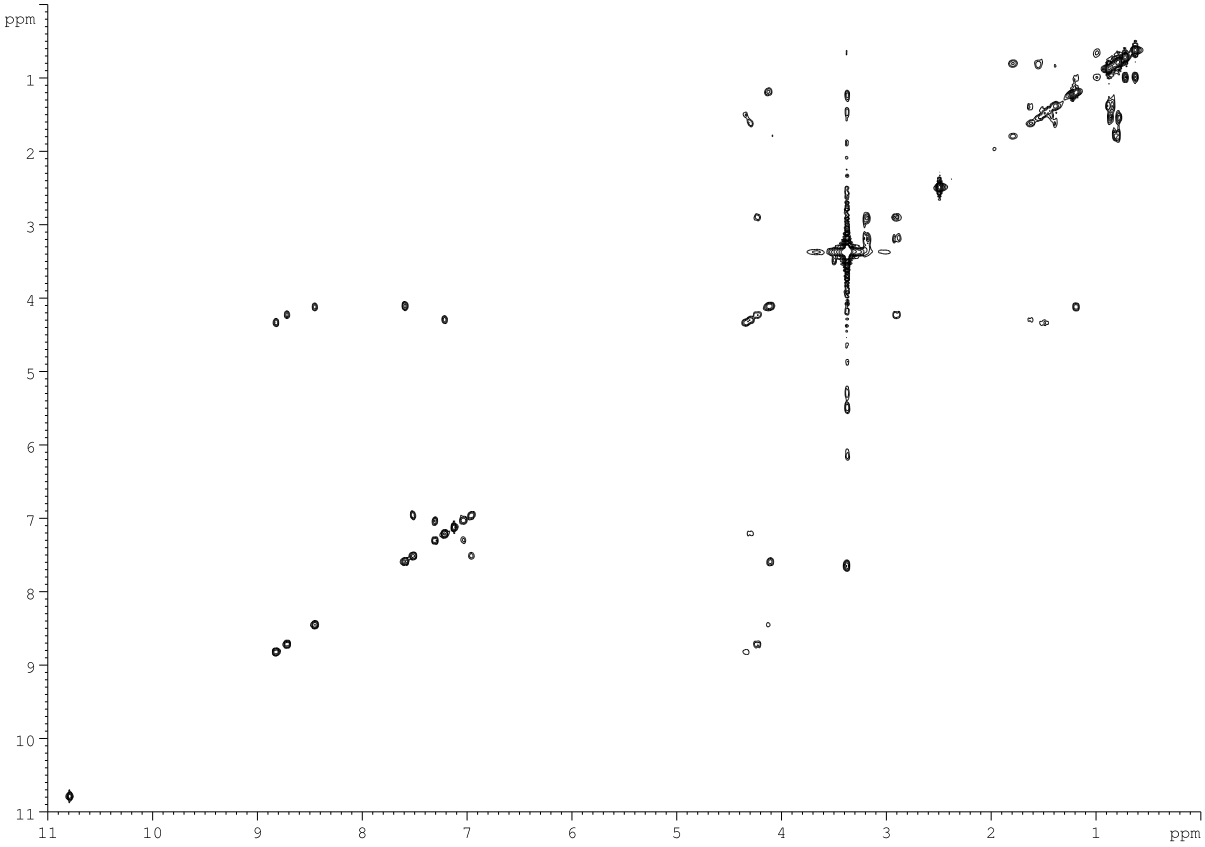


Figure S46. ^{1}H, ^{1}H COSY spectrum of **12** in DMSO-d_6

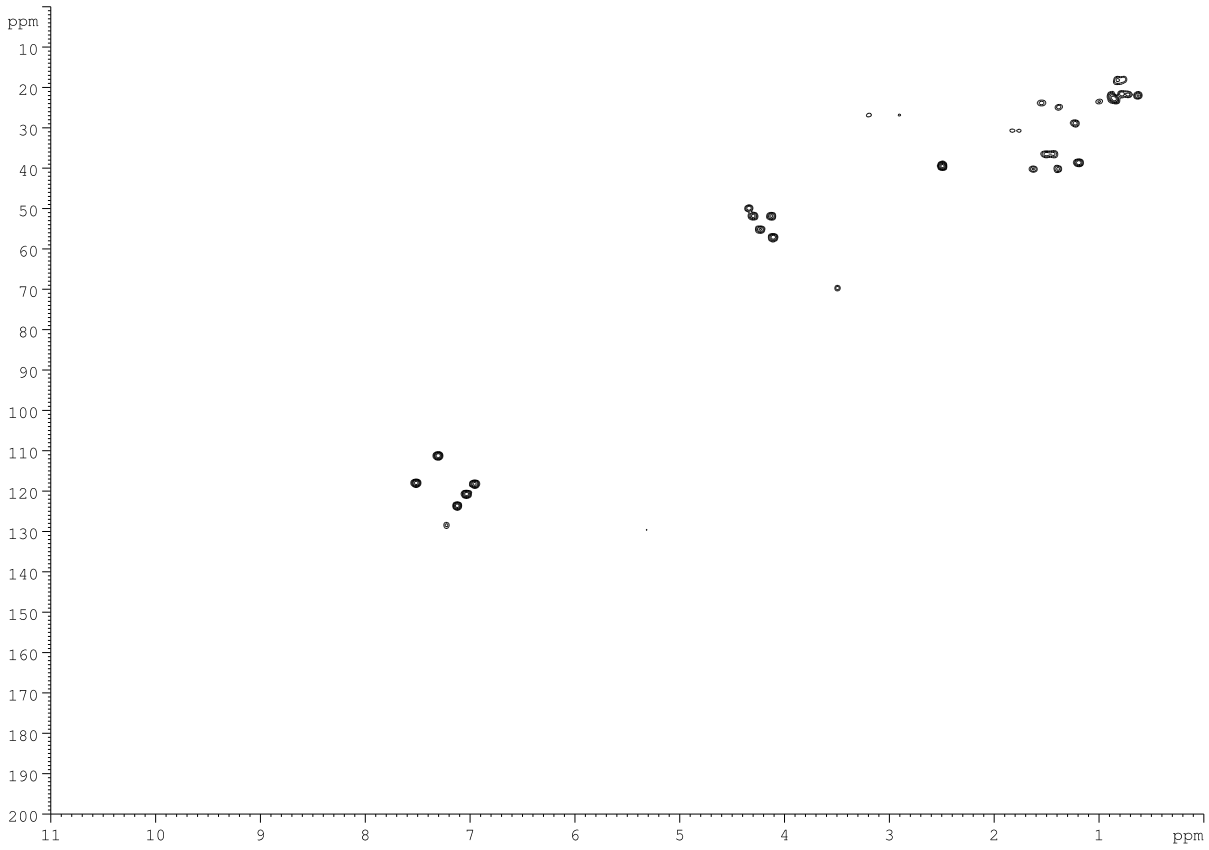


Figure S47. ^{1}H, ^{13}C HSQC spectrum of **12** in DMSO-d_6

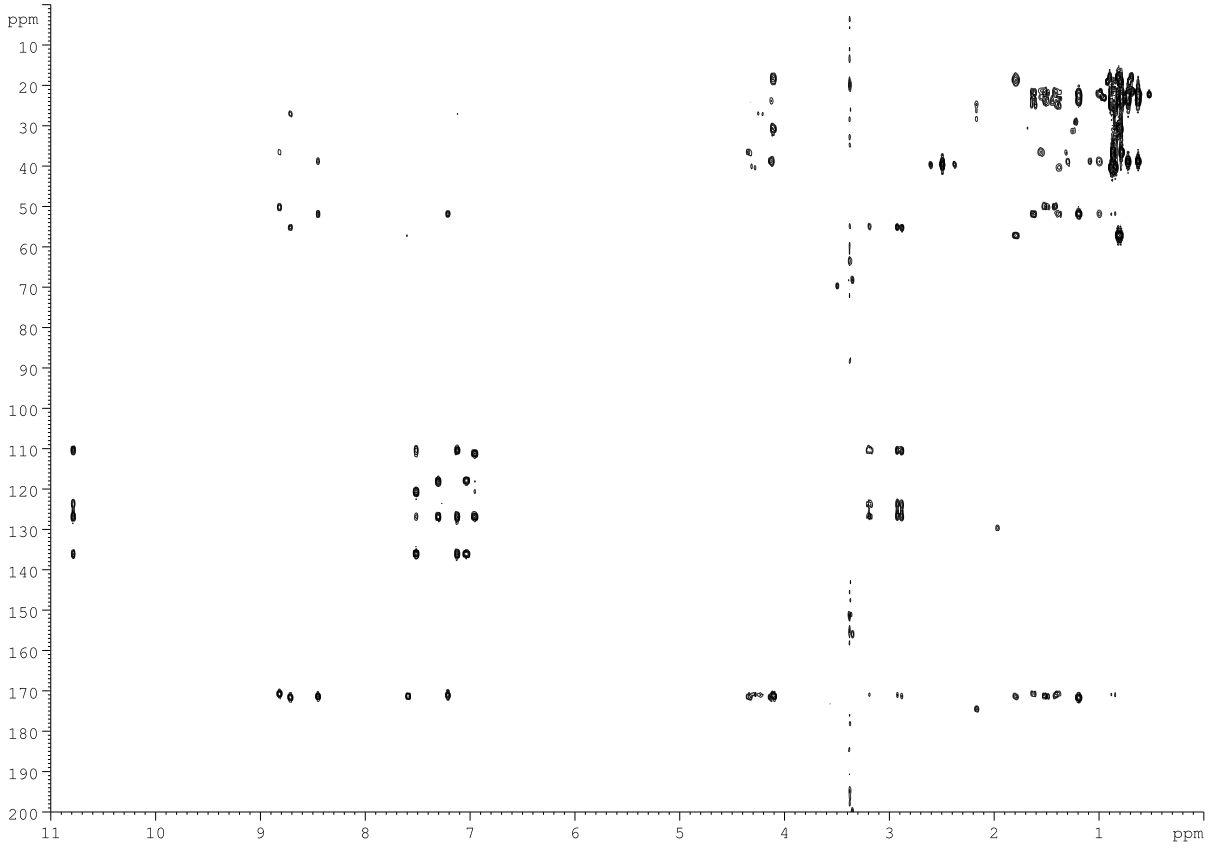


Figure S48. ^{1}H, ^{13}C HMBC spectrum of **12** in DMSO-d_6

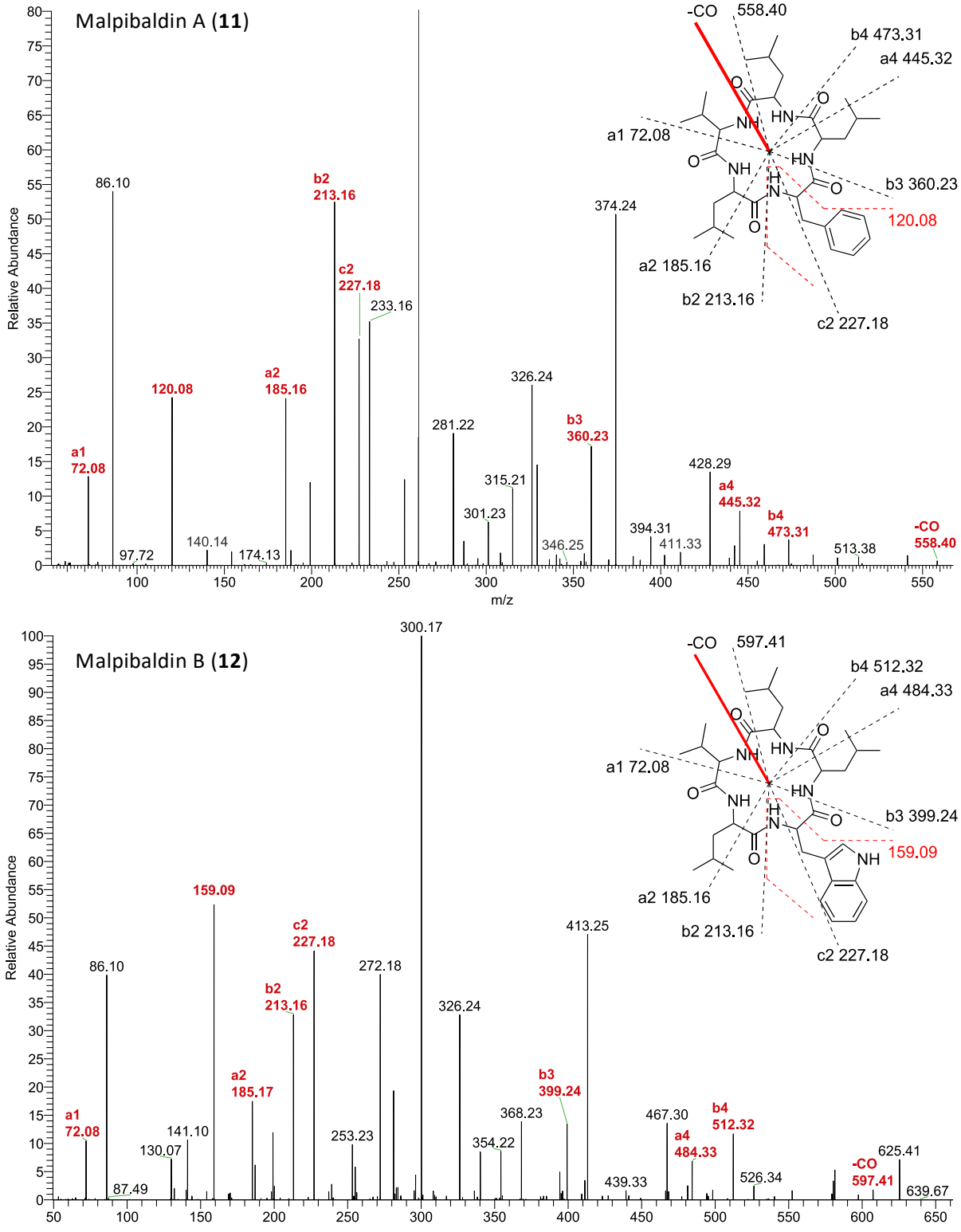


Figure S49. ESI-MS-MS-data of **11** and **12** with most likely ion fragments of the proposed structures.

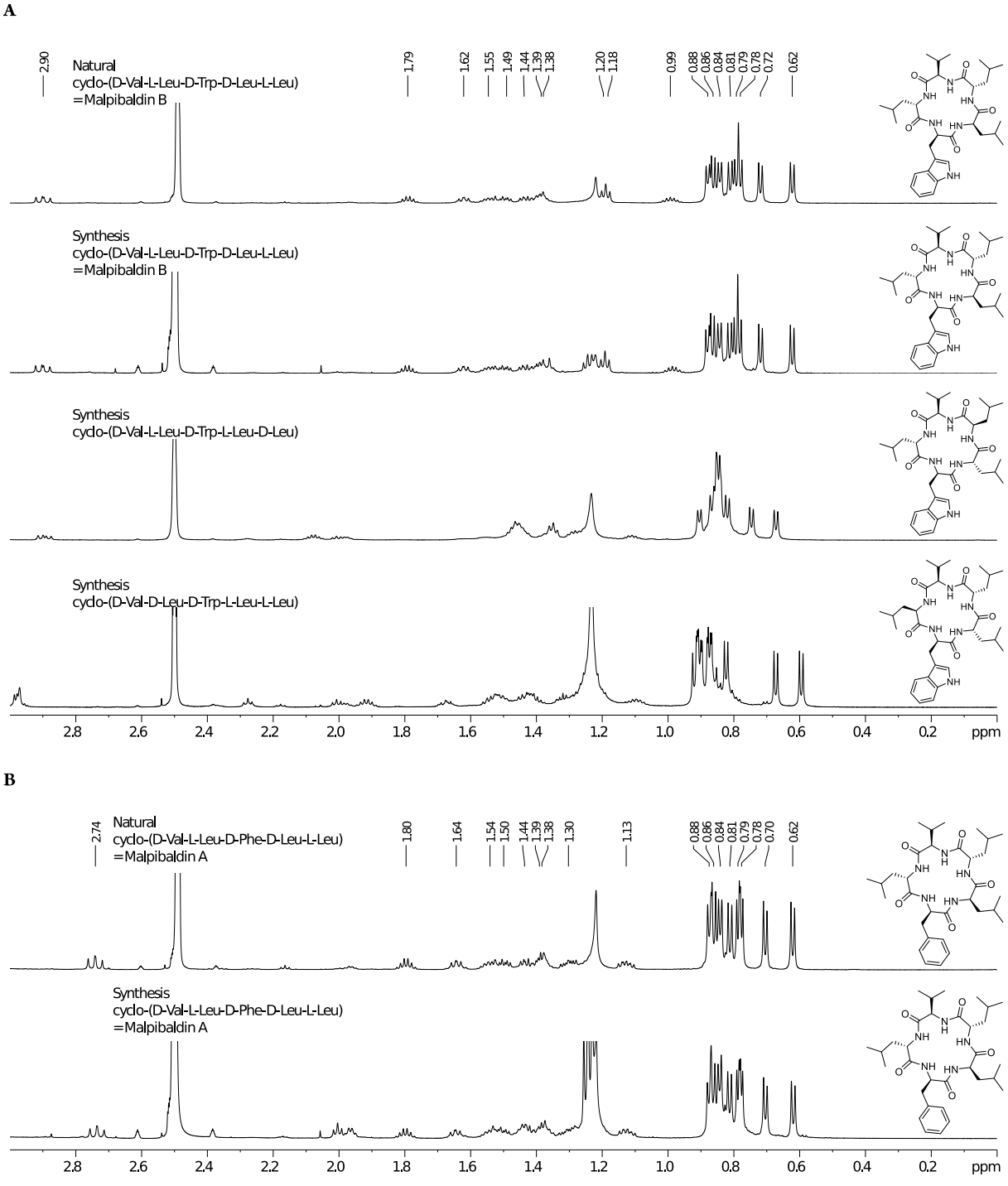


Figure S50. Comparison of ^{1}H NMR spectra of synthetic stereoisomers of **11** and **12**. (**A**) Comparison of three synthetic stereoisomers of **12** with permutated L- and D-configured leucine moieties. (**B**) Comparison of the natural and synthetic **11**. The configuration determines the chemical shifts and coupling constants of the methyl groups of the leucine moieties (Tab. S5).

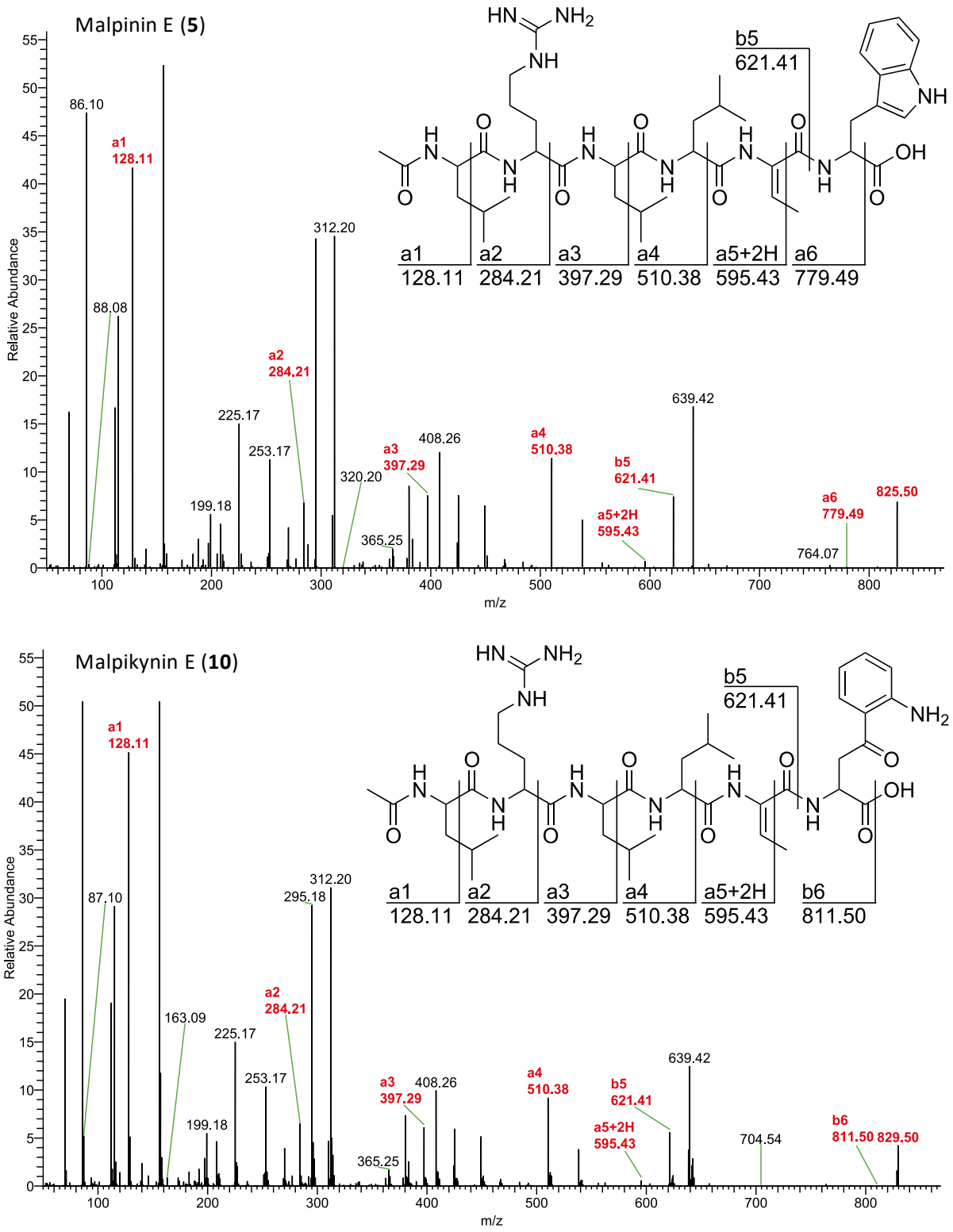


Figure S51. ESI-MS-MS-data of **5** and **10** with most likely ion fragments of the proposed structures.

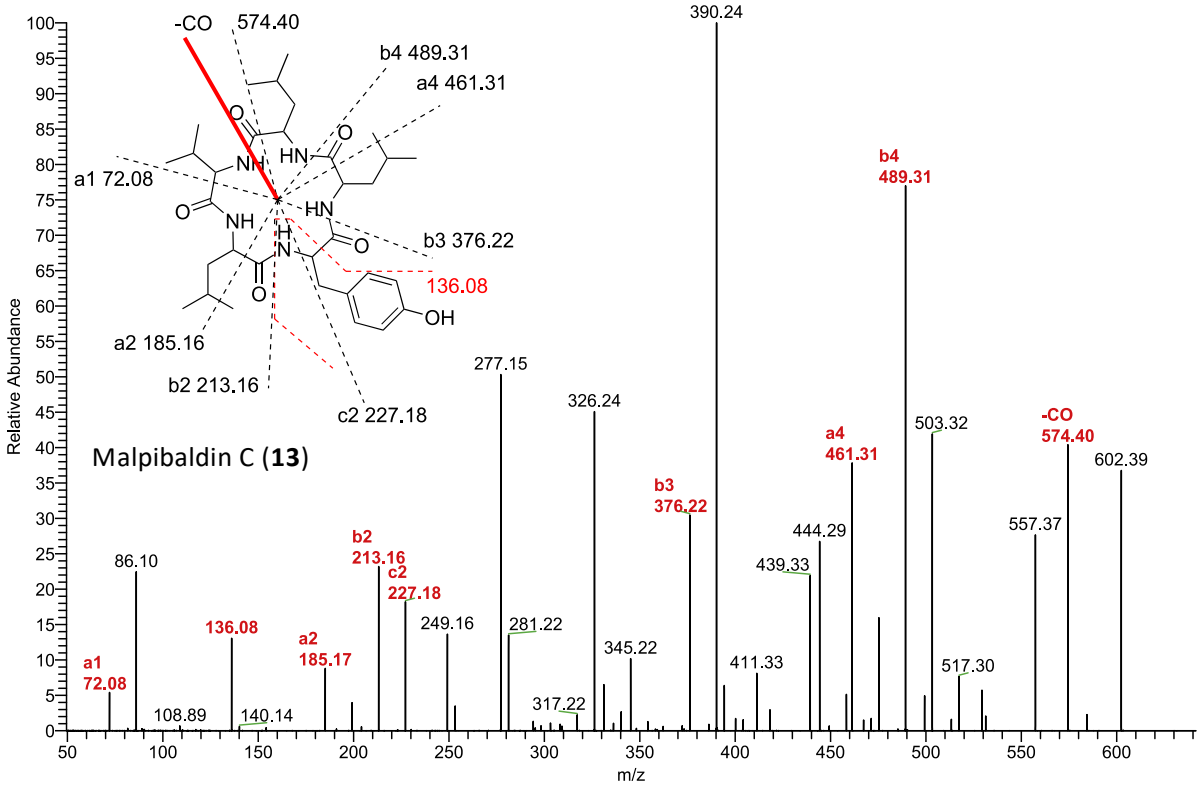


Figure S52. ESI-MS-MS-data of **13** with most likely ion fragments of the proposed structures.

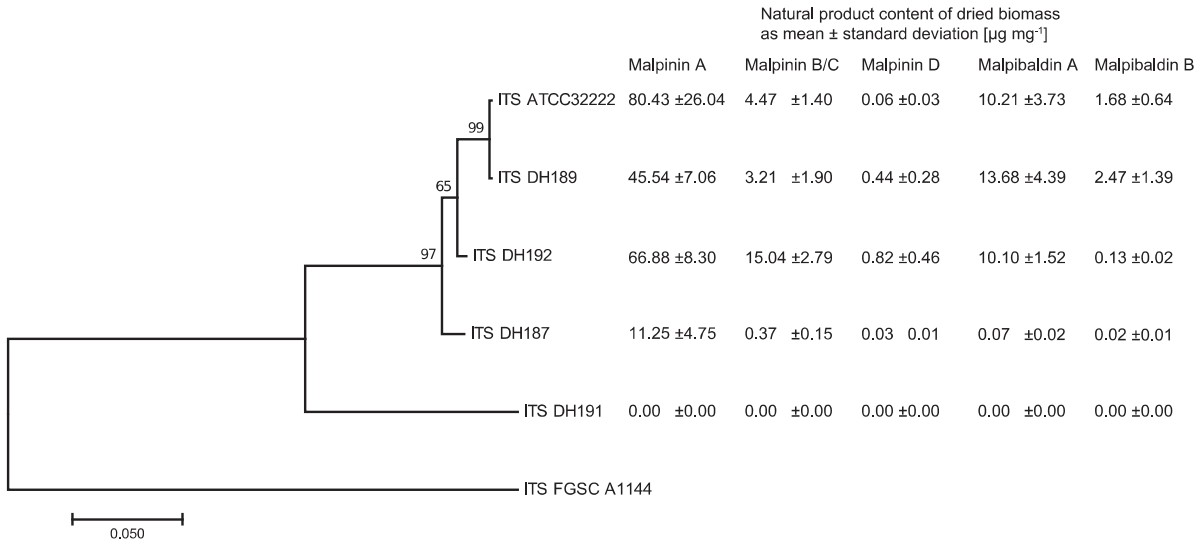


Figure S53. Phylogenetic analysis and metabolite production in *M. alpina* strains ATCC32222, DH187, DH189, DH192 and *M. cystojenkinii* strain DH191. (**A**) The phylogenetic analysis based on the internal transcribed spacer (ITS) sequence was inferred by using the Maximum Likelihood method based on the Tamura-Nei model.[9] The percentage of trees in which the associated taxa clustered together is shown next to the branches (bootstrap values). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7.[2] The ITS sequence of the ascomycete *Aspergillus niger* FGSC A1144 served as outgroup. (**B**) Production of malpinins and malpibaldins in *Mortierella alpina* strains were calculated as µg metabolite per mg fungal dry weight according to a metabolite calibration standard.

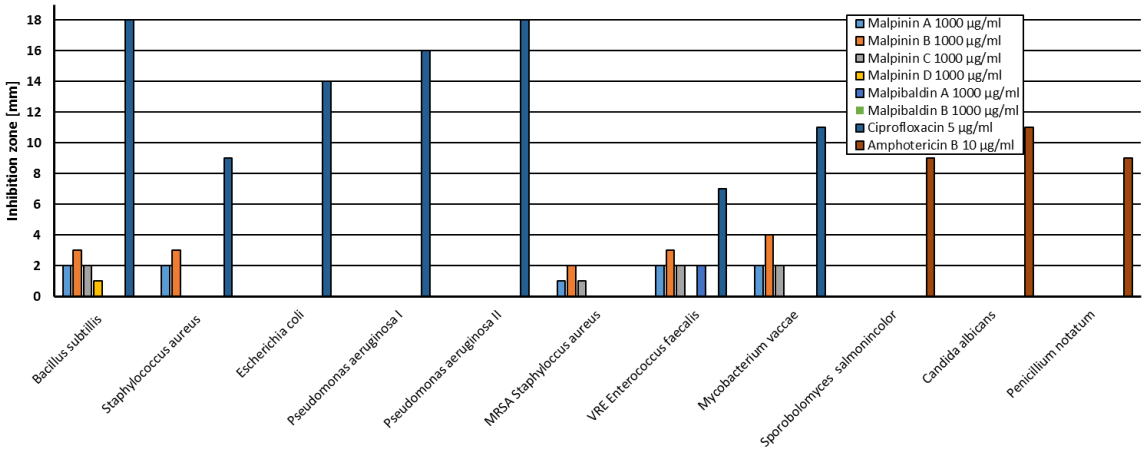


Figure S54. Antimicrobial activity of **1 - 4** and **11 – 12.** Apart from a slight inhibition of Gram-positive bacteria in the case of malpinins, none of the metabolites show severe antimicrobial activity. Ciprofloxacin and amphotericin B served as antibacterial and antifungal positive controls, respectively.

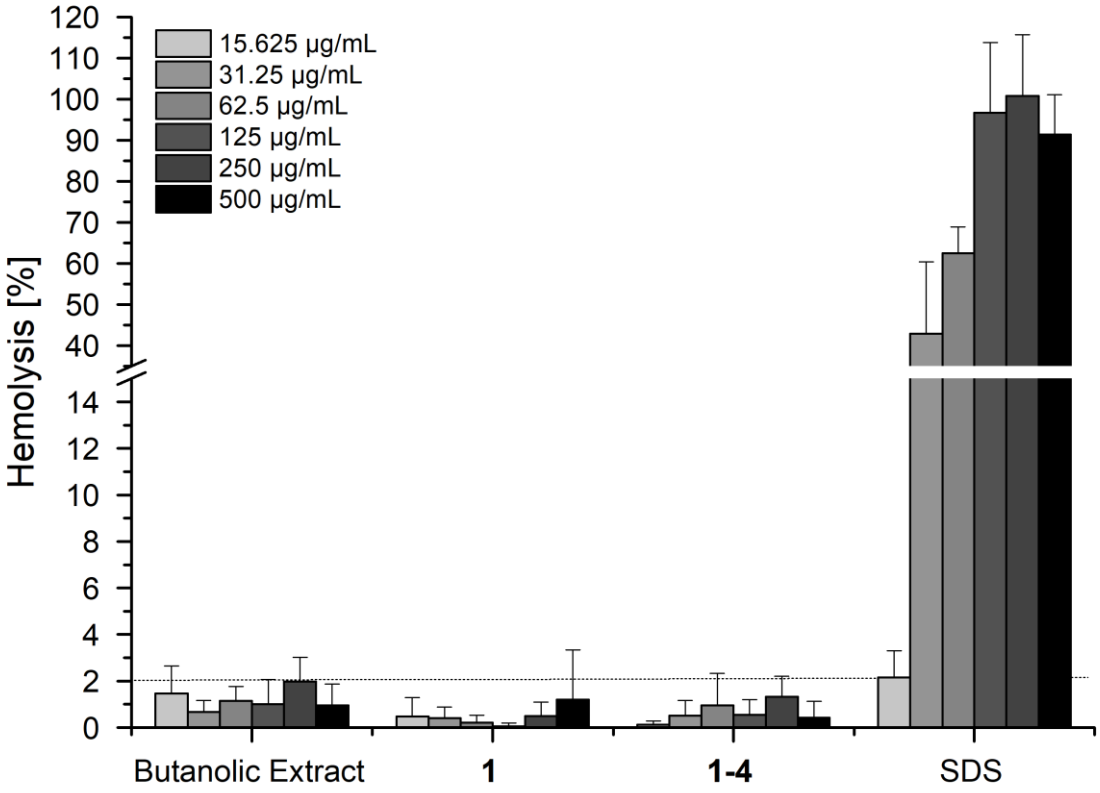


Figure S55. Hemolytic activity of butanolic extracts, **1** – **4** and **1** compared to SDS**.**

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