SUPPLEMENTARY MATERIAL

Dithiodiketopiperazine derivatives from endophytic fungi *Trichoderma harzianum* and *Epicoccum nigrum*

Harwoko Harwoko[a,b], Georgios Daletos[a], Fabian Stuhldreier[c], Jungho Lee[d,e], Sebastian Wesselborg[c], Michael Feldbrügge[d,e], Werner E. G. Müller[f], Rainer Kalscheuer[a], Elena Ancheeva[a,*] and Peter Proksch[a,*]

[a]*Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Universitaetsstrasse 1, Geb. 26.23, 40225 Duesseldorf, Germany;* [b]*Department of Pharmacy, Faculty of Health Sciences, Universitas Jenderal Soedirman, Jalan dr. Soeparno, Karangwangkal 53123 Purwokerto, Indonesia;* [c]*Institute of Molecular Medicine I, Medical Faculty, Heinrich Heine University, Universitaetsstraße 1, Geb. 23.12, 40225 Duesseldorf, Germany;* [d]*Institute for Microbiology, Heinrich Heine University, Universitaetsstrasse 1, Geb. 26.12, 40225 Duesseldorf, Germany;* [e]*Bioeconomy Science Center (BioSC), c/o Forschungszentrum Juelich, 52425 Juelich, Germany;* [f]*Institute of Physiological Chemistry, Universitaetsmedizin der Johannes Gutenberg-Universitaet Mainz, 55128 Mainz, Germany*

Corresponding authors:

* Prof. Dr. Peter Proksch, Email: proksch@uni-duesseldorf.de, Tel.: +49-211-81-14163, Fax.: +49 211 81 11923.

* Dr. Elena Ancheeva, Email: elena.ancheeva@uni-duesseldorf.de, Tel.: +49-211-81-14173, Fax.: +49 211 81 11923.

Abstract

A new epidithiodiketopiperazine (ETP), pretrichodermamide G (**1**), along with three known (epi)dithiodiketopiparazines (**2**-**4**) were isolated from cultures of *Trichoderma harzianum* and *Epicoccum nigrum*, endophytic fungi associated with medicinal plants *Zingiber officinale* and *Salix* sp., respectively. The structure of the new compound (**1**) was established on the basis of spectroscopic data, including 1D/2D NMR and HRESIMS. The isolated compounds were investigated for their antifungal, antibacterial and cytotoxic potential against a panel of microorganisms and cell lines. Pretrichodermamide A (**2**) displayed antimicrobial activity towards the plant pathogenic fungus *Ustilago maydis* and the human pathogenic bacterium *Mycobacterium tuberculosis* with MIC values

of 1 mg/mL (2 mM) and 25 μg/mL (50 μM), respectively. Meanwhile, epicorazine A (**3**) exhibited strong to moderate cytotoxicity against L5178Y, Ramos, and Jurkat J16 cell lines with IC_{50} values ranging from 1.3 to 28 μM. Further mechanistic studies indicated that **3** induces apoptotic cell death.

Keywords: *Trichoderma harzianum*; *Epicoccum nigrum*; epidithiodiketopiperazine; endophytic fungi; antimicrobial activity; cytotoxicity; apoptosis.

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Experimental section

General experimental procedures

HPLC analysis was performed with a Dionex p580 DAD3000RS (Dionex Softron, Munich, Germany) with a LPG-3400SD pump coupled with a photodiode array detector (UVD340S), using routine detection channels at 235, 254, 280, and 340 nm wavelengths. ^{1}H-NMR (600 MHz), ^{13}C-NMR (150 MHz), and 2D NMR spectra were recorded at 297.9°K in DMSO-d_6 Bruker Avance III 600 MHz NMR spectrometers (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts are in ppm referring to the deuterated solvent peaks at δ_H 2.50 and δ_C 39.5 (DMSO-d_6) for ^{1}H and ^{13}C, respectively. Mass spectra were measured by a HP110 Agilent Finnigan LCQ Deca XP Thermoquest mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), while high resolution mass spectra (HRESIMS) were recorded on a UHR-TOF maxis 4G mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A Jasco P-2000 digital polarimeter (Jasco International, Tokyo, Japan) was used for determination of specific optical rotation. All solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements.

The separation column (125×4 mm, L×ID) was prefilled with Eurosphere-10C_{18} (Knauer, Germany), and the following gradient was used (MeOH, 0.1% formic acid in H_2O): 0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH). Semi-preparative RP-HPLC was performed using a Merck Hitachi system (UV detector L-7400; pump L-7100; Eurosphere-100C_{18}, 300×8 mm, Merck KGaA, Darmstadt, Germany). Column chromatography included LH-20 Sephadex, and Merck silica gel 60 M (0.04 - 0.063 mm). TLC plates pre-coated with silica gel 60 F_{254} (Macherey-Nagel, Dueren, Germany) were used to monitor fractions, followed by detection under UV 254 and 366 nm or after spraying with anisaldehyde-sulfuric acid reagent.

Fungal material

The fungus *Trichoderma harzianum* was isolated from fresh and healthy leaves of *Zingiber officinale*, collected in Banyumas, Central Java, Indonesia, in May 2016. *Epicoccum nigrum* was isolated from fresh and healthy leaves of *Salix* sp., collected in Lago Naki, the Republic of Adygea (North Caucasus), Russia, in May 2017. Isolation of fungi was achieved by the dilution plate method employing isolation medium (15 g/L bacto agar, 15 g/L malt extract in distilled water, at pH 7.4–7.8) supplied by chloramphenicol (0.20 g/L) and streptomycin sulfate (0.25 g/L) in order to inhibit the growth of bacteria and actinomycetes.

Fungal identification was performed according to a molecular biology protocol by DNA amplification and sequencing of the ITS region with GenBank accession No. MK213940 and MK214079 for *T. harzianum* and *E. nigrum*, respectively (Kjer et al. 2010). Voucher strains were deposited at the corresponding authors' laboratory (P.P.).

Fermentation and isolation

The fungal strains were cultivated on solid rice medium, which was prepared by autoclaving 100 g of rice and 110 mL of water in a 1 L Erlenmeyer flask, except for compound **4** isolated from *E. nigrum* cultivated on green lentil medium which was prepared in the same manner. Large scale fermentation of these two fungi was performed in five flasks for each fungus for 14 days at room temperature under static condition. The cultures were diced and extracted with EtOAc (5 L). The crude extract of *T. harzianum* (11.34 g) was partitioned by liquid-liquid extraction using 90%MeOH-H_2O and *n*-hexane to yield the MeOH fraction (4.40 g), which was then subjected to vacuum liquid chromatography (VLC) using a step gradient of *n*-hexane/EtOAc, followed by DCM/MeOH, to yield 15 fractions (F1-F15). Fractions 7 and 8 (F78, 0.49 g), eluted with DCM/MeOH (95/90:5/10), were subjected to size exclusion chromatography using a Sephadex LH-20 column (100×2.5 cm) with 50%DCM-MeOH as eluting solvent. The subfractions HTH-V78-SD1-2-3 containing ETPs (TLC monitoring) were selected for further purification via semi-preparative RP-HPLC using gradient elution of water and methanol yielding compounds **1** (8.84 mg) and **2** (22.69 mg). The crude extract of *E. nigrum* (4.5 g) was subjected to VLC using n-hexane/EtOAc (10/90) to yield fraction F5, then F5 subjected to a size exclusion chromatography using a Sephadex LH-20 column with 100% methanol as eluting solvent to yield six subfractions. Two subfractions (HEN-V5-D4/5) were further purified by semi-preparative RP-HPLC to yield compounds **3** (0.50 mg) and **4** (2.0 mg).

*Pretrichodermamide G (**1**)*

yellow solid; $[\alpha]^{20}_{D}$: − 103.7 (*c* 0.17, MeOH); UV λ_{max} (MeOH) 203, 283 nm; ^{1}H and ^{13}C NMR spectral data in DMSO-d_6, see Table S1; HRESIMS (+) *m/z* 499.0828 $[M + H]^+$ (calcld for $C_{20}H_{22}N_2O_9S_2$, 499.0839, Δ 1.1 mmu).

Biological assay

Antibacterial assay

The antibacterial assay was carried out using the broth microdilution method following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2012).

A panel of Gram-positive and Gram-negative bacterial strains, including *Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli* as well as *Mycobacterium tuberculosis* H_{37}Rv were selected for this evaluation.

Antifungal assay

The antifungal assay was performed using the agar diffusion (Kirby-Bauer) method. *Ustilago maydis* AB33 (Brachmann et al. 2001) and *Saccharomyces cerevisiae* ESM356-1 (Pereira et al. 2001) were grown in CM medium supplemented with 10 g/L glucose (Holliday, 1974; Banuett and Herskowitz, 1989) and YPD respectively. Sterile filter paper disks of 5 mm diameter were placed on agar plates previously inoculated with *U. maydis* or *S. cerevisiae*. The disks were then impregnated with 100 µg of the compounds, which were dissolved in DMSO. Double distilled water (ddH_2O) and DMSO were used as negative controls, while nystatin (100 µg) and nourseothricin (200 µg) were used as positive controls. The treated agar plates were incubated at 28°C for 48 h, afterwards antifungal activity was recorded as growth inhibition zones (in mm) of inhibition surrounding the disk. Antifungal assay against *Candida albicans* was performed by microdilution method with the same protocol as antibacterial assay. The test compound was considered active when the growth of inhibition zone was greater than 8 mm, while the lowest concentration at which no observed growth inhibition zone was taken to be the minimum inhibitory concentration (MIC). Natural products and controls were tested in triplicate apart from epicorazine A which was tested once due to the limited amount of this compound.

Cytotoxicity and apoptosis assays

Cytotoxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay employing mouse lymphoma cells (L5178Y), lymphoblastic leukemia T cells (Jurkat J16, no. ACC-282), Burkitt's lymphoma B cells (Ramos, no. ACC-603), and human ovarian cancer cells (A2780 sens). Kahalalide F and staurosporine (STS) were used as positive controls and media with 0.1% DMSO as negative control. All experiments were carried out in triplicate and performed as described earlier (Liu et al. 2017). With regard to the observed cytotoxicity of compound **3**, we subsequently evaluated the potential contribution of pro-apoptotic mechanisms through two different methods: (1) western blotting of the caspase-3 substrate PARP and (2) measuring the increase in fluorescence of the pro-fluorescent caspase-3 substrate Ac-DEVD-AMC. Caspase activity

was determined as the slope of the resulting linear regressions. Data points shown are the mean of triplicates, error bars = SD. Values are normalized to DMSO (0.1% v/v) treated cells (=100 %).

Table S1. NMR spectroscopic data of **1** recorded at 600 (^{1}H) and 150 (^{13}C) MHz (DMSO-d_6, δ in ppm).

Position	**δ_C, type***	**δ_H, mult (*J* in Hz)**	**COSY**	**ROESY**	**HMBC**
1	166.7, C	-			
2	68.0, C	-			
3a	37.9, CH_2	2.10, d (15.0)	3b	5, 4-OH	1, 2, 4, 5, 9
3b		2.36, d (15.0)	3a	5, 6	1, 2, 4, 5
4	70.8, C	-			
5	133.4, CH	5.53, d (10.0)	6	3ab, 4-OH, 7	7, 9
6	127.7, CH	5.60, dd (10.0; 4.2)	5, 7		4
7	65.8, CH	4.04, q (4.8)	6, 7-OH, 8	5	
8	66.3, CH	3.78, ddd (8.8, 6.5, 4.8)	7, 8-OH, 9	3b	
9	82.2, CH	4.13, d (8.8)	8	4-OH	
1’	164.8, C	-			
2’	59.0, CH	4.37, dd (4.7, 2.1)	10’-NH	3’, 5’	1’, 3’
3’	44.9, CH	4.49, d (2.1)		2‘, 9-OH, 10’-NH	1’, 2’, 4’, 5’, 9’
4‘	116.2, C	-			
5‘	122.6, CH	7.43, d (8.8)	6‘	2‘	3‘, 7‘, 9‘
6‘	103.1, CH	6.55, d (8.8)	5‘	7‘-OCH_3	4‘, 7‘, 8‘
7‘	153.1, C	-			
8’	136.0, C	-			
9’	147.6, C	-			
10’-NH	-	8.97, d (4.7)	2’	3‘	1‘, 2
7‘-OCH_3	CH_3	3.78, s		6‘	7‘
8‘-OCH_3	CH_3	3.67, s		9’-OH	8‘
4-OH	-	5.27, s		3a, 5, 9	3, 4, 9
7-OH	-	4.95, d (5.5)	7		6
8-OH	-	4.48, d (6.5)	8		
9’-OH	-	9.47, s		3’, 8’-OCH_3	9, 4‘, 8‘

* Data extracted from HSQC and HMBC spectra

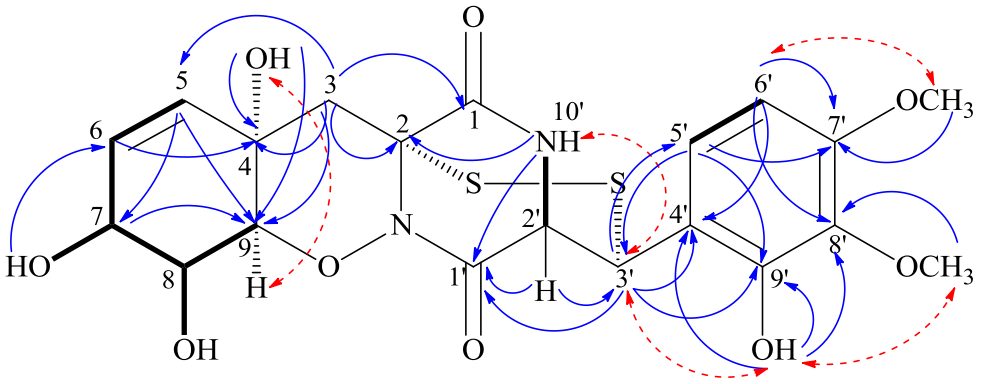


Figure S1. COSY (bold), HMBC (blue line) and key ROESY (red dashed) correlations of compound **1**.

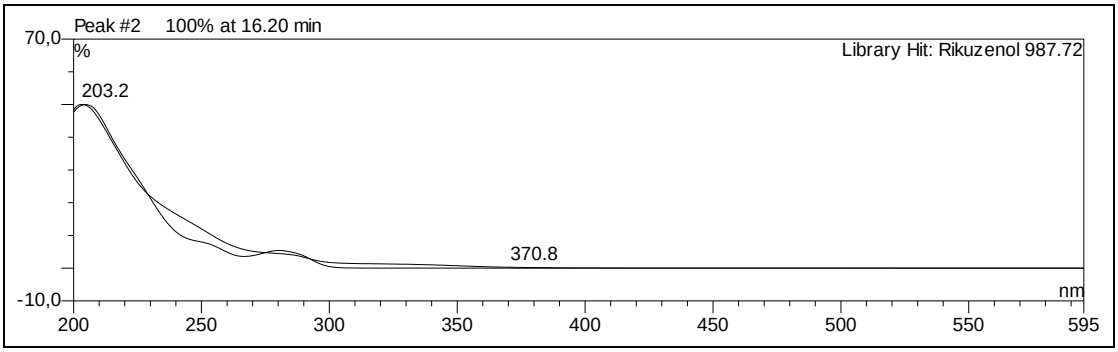
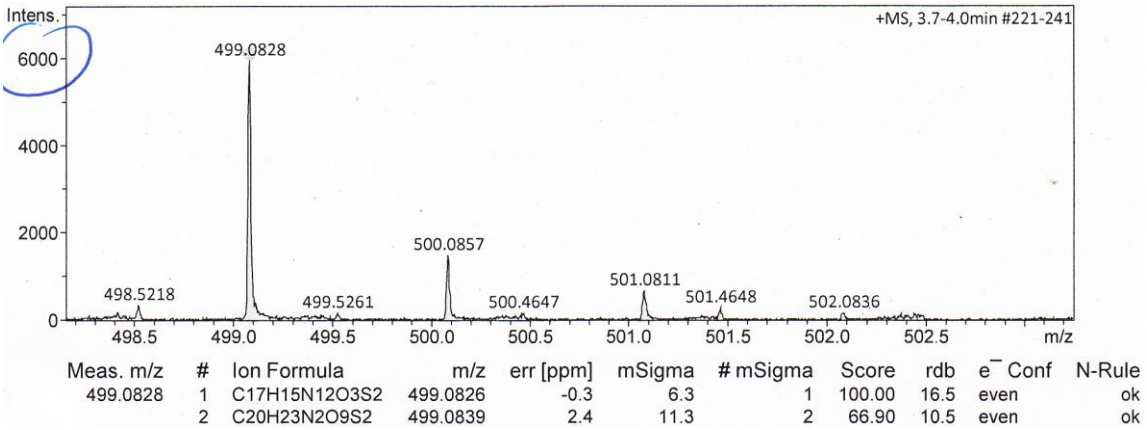


Figure S2. UV spectrum of compound **1**.



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule
499.0828	1	C17H15N12O3S2	499.0826	-0.3	6.3	1	100.00	16.5	even	ok
	2	C20H23N2O9S2	499.0839	2.4	11.3	2	66.90	10.5	even	ok

Figure S3. HRESIMS spectrum of compound **1**.

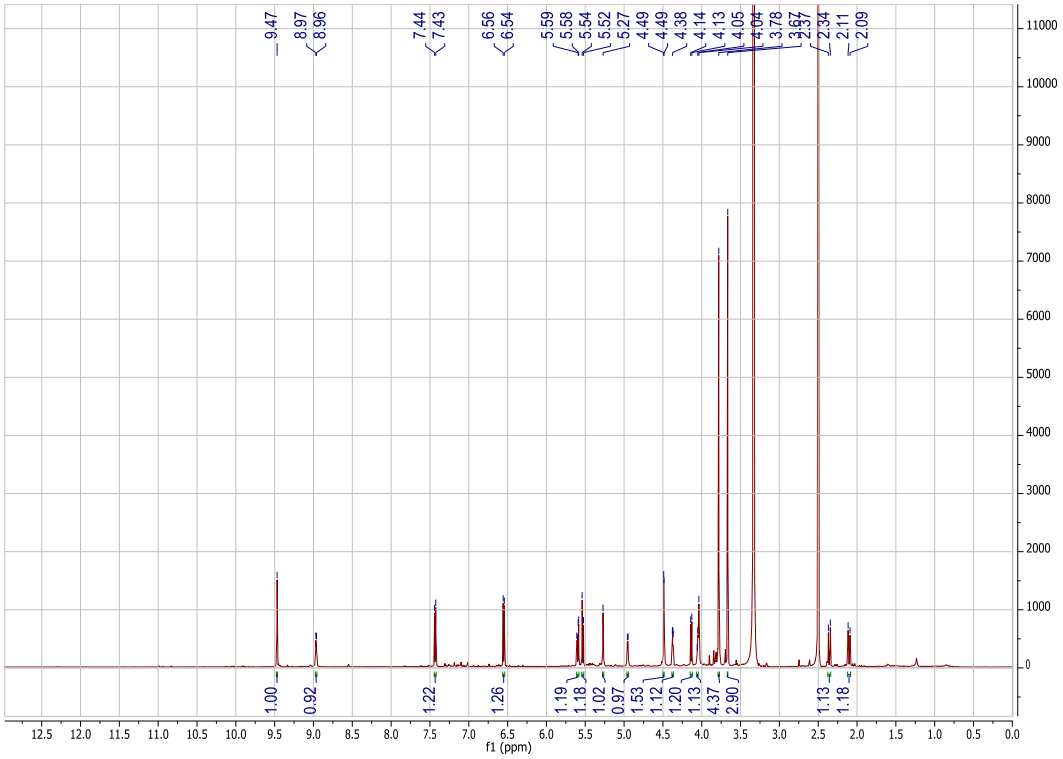


Figure S4. ^{1}H NMR (600 MHz, DMSO-d_6) spectrum of compound **1**.

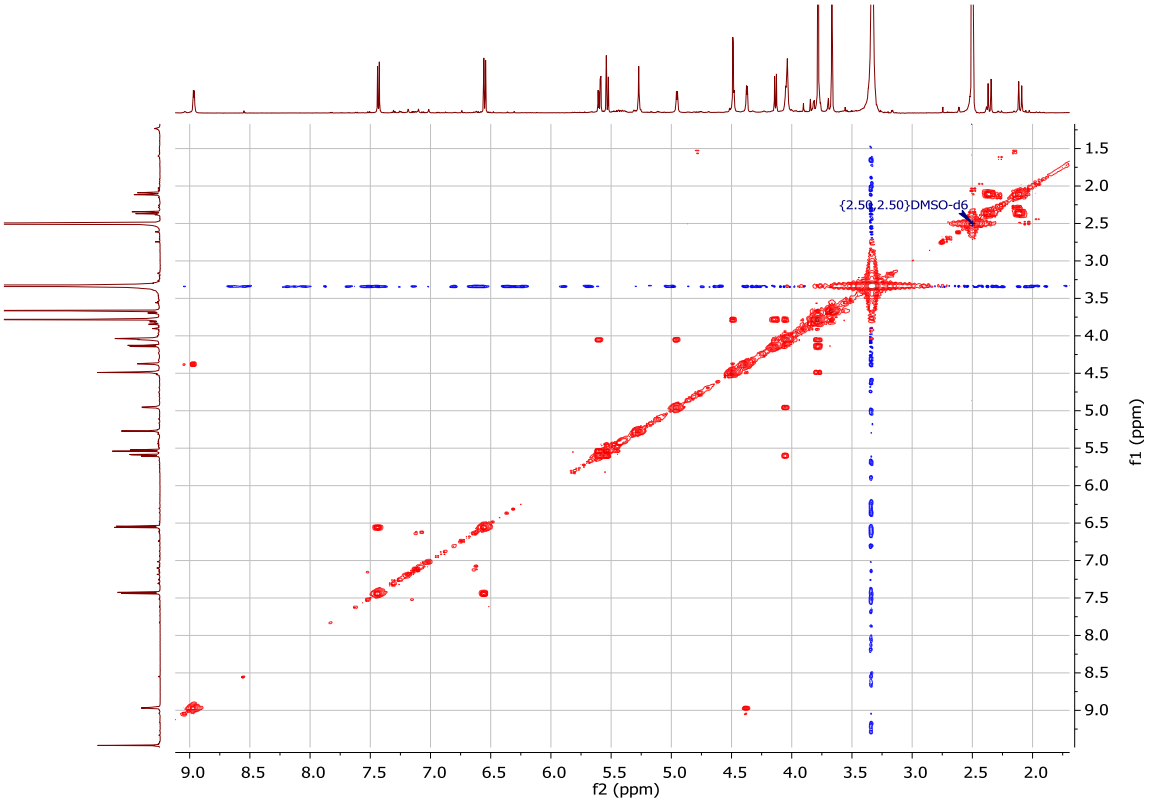


Figure S5. ^{1}H-^{1}H COSY (600 MHz, DMSO-d_6) spectrum of compound **1**.

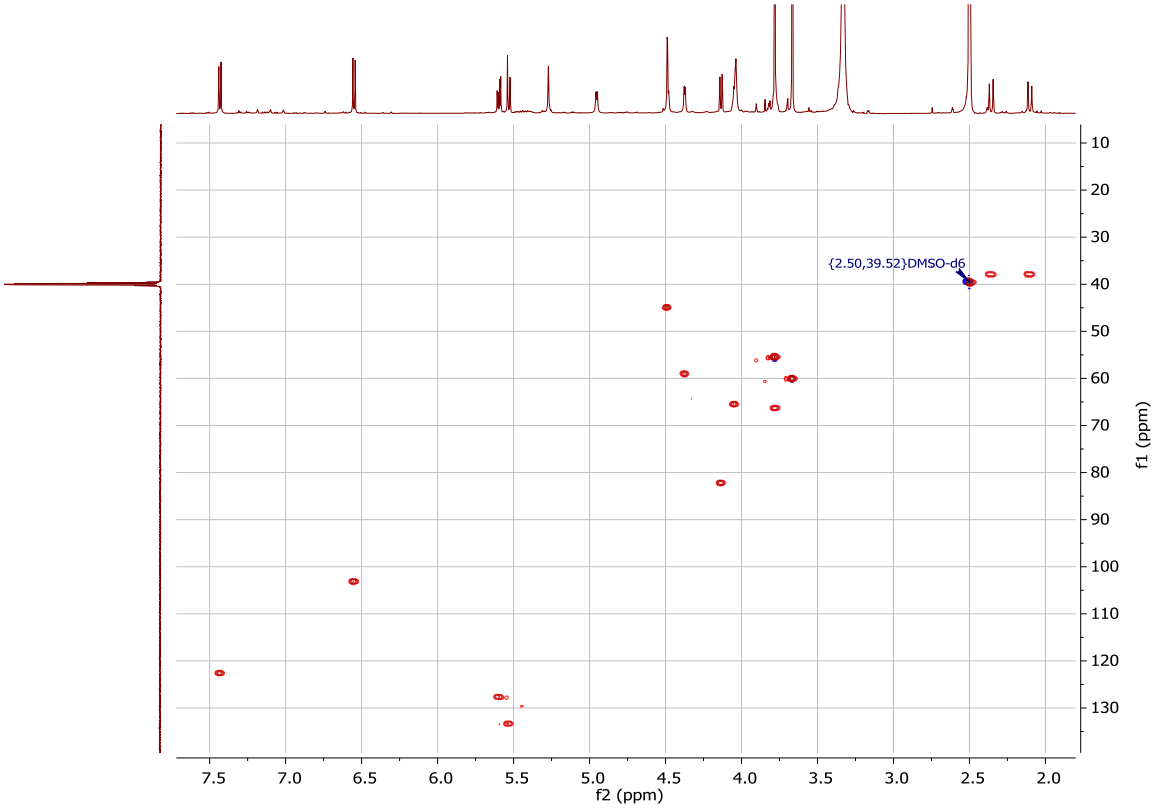


Figure S6. HSQC (600 and 150 MHz, DMSO-d_6) spectrum of compound **1**.

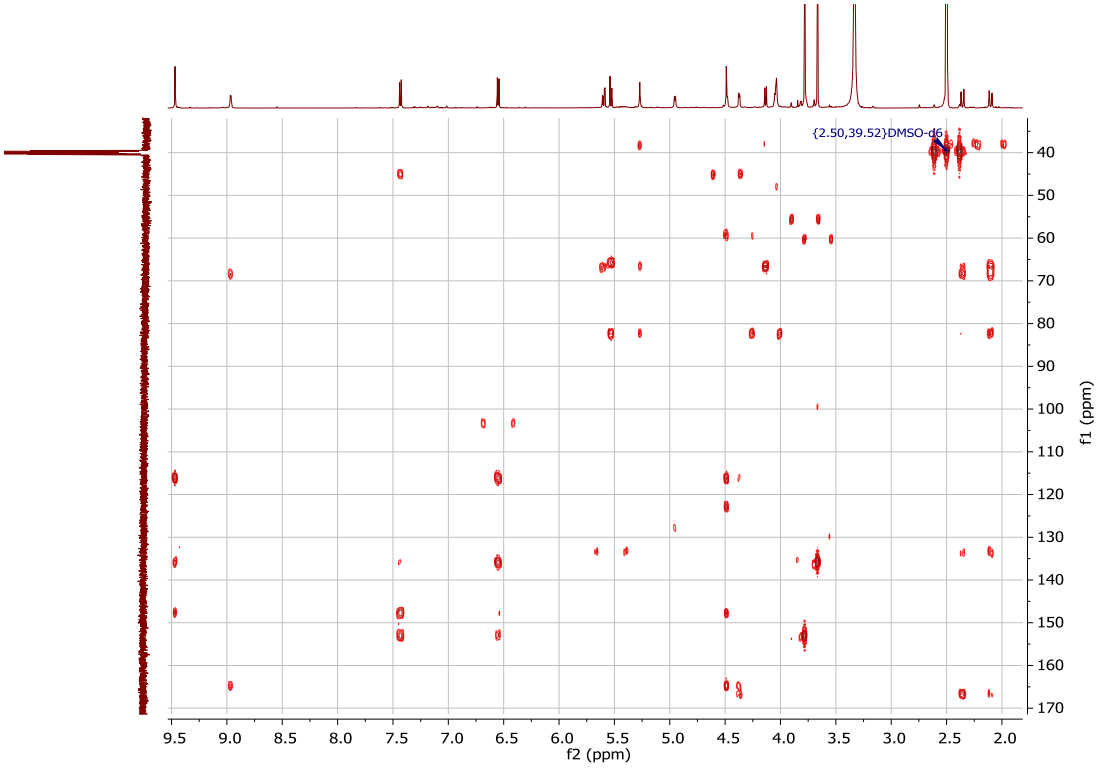


Figure S7. HMBC (600 and 150 MHz, DMSO-d_6) spectrum of compound **1**.

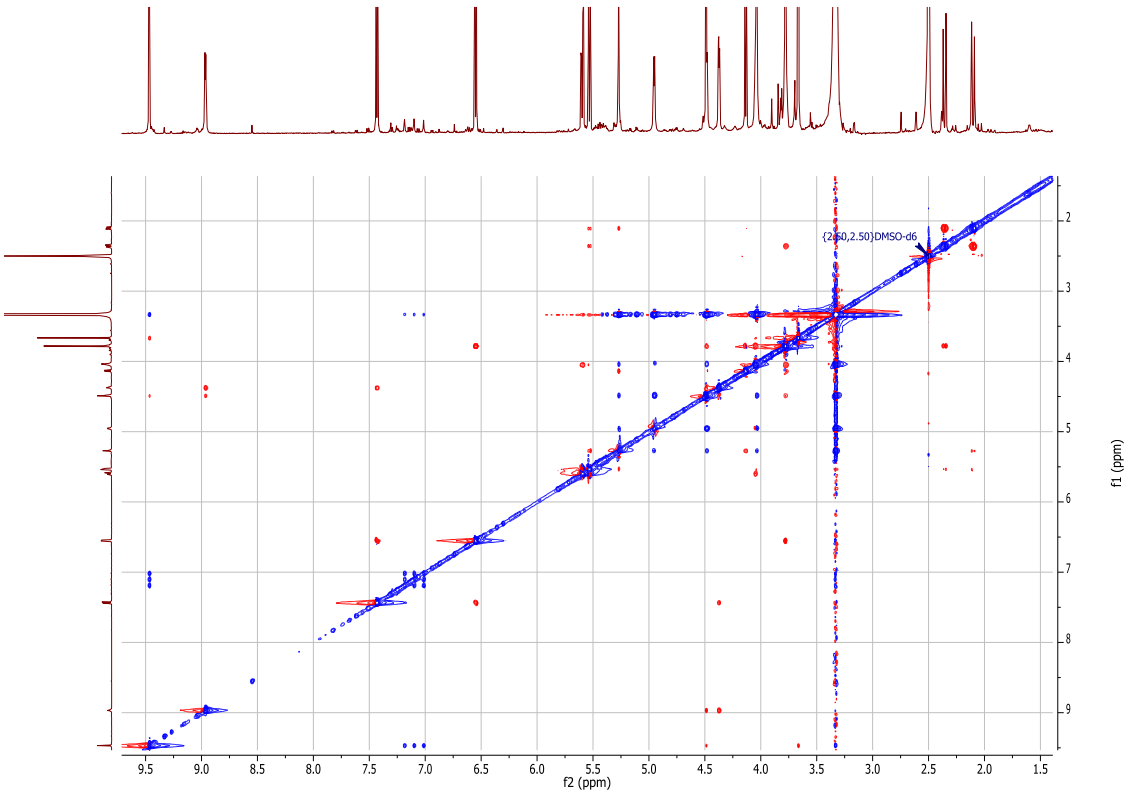


Figure S8. ROESY (600 MHz, DMSO-d_6) spectrum of compound **1**.

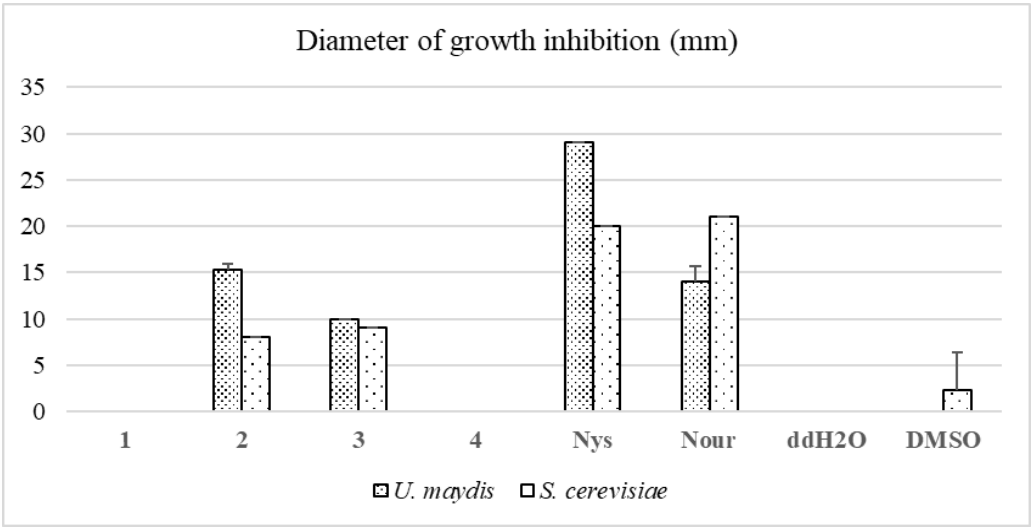


Figure S9. Diameter of growth inhibition (mm) towards *Ustilago maydis* and *Saccharomyces cerevisiae* of pretrichodermamide G (**1**), pretrichodermamide A (**2**), epicorazine A (**3**), and ent-epicoccin G (**4**), along with nystatin (Nys) and nourseothricin (Nour) as well as double distilled water (ddH_2O) and DMSO.

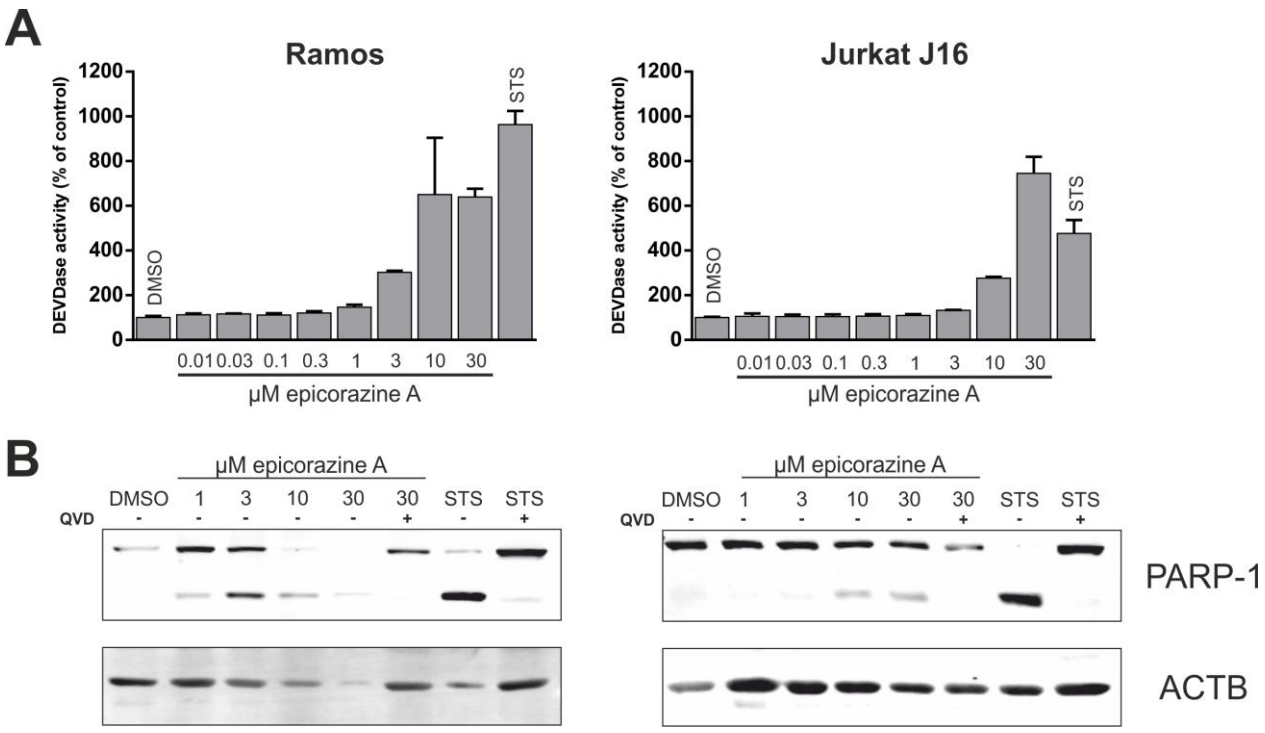


Figure S10. Epicorazine A (**3**) induces apoptosis.

(A) Ramos cells (Burkitt's lymphoma B lymphocytes) and Jurkat J16 cells (acute T cell leukemia cells) were treated with the indicated concentrations of epicorazine A (**3**) or the positive control staurosporine (2.5 µM) for 24 h. Afterwards the cells were lysed and loaded with the pro-fluorescent caspase-3 substrate Ac-DEVD-AMC and fluorescence of AMC was measured over the course of 2 h in a microplate reader. Increase in fluorescence (DEVDase activity) is considered as activation of pro-caspase-3. (B) Ramos and Jurkat J16 cells were treated with the indicated concentrations of epicorazine A (**3**) in the absence or presence of the caspase inhibitor Q-VD-OPh (10 µM) for 24 h. Cells treated with staurosporine (STS, 2.5 µM) were used as positive control for caspase-dependent cleavage of Poly (ADP-ribose) polymerase-1 (PARP). After incubation period, cleavage of PARP was determined by Western blot analysis. The expression of Beta-actin was determined as protein loading control. Figure S10B shows the result of a representative blot.

Table S2. Minimum inhibitory concentrations (μg/mL) of the isolated compounds **1-4**

Compound	*M. tuberculosis*	*Candida albicans*	*Ustilago maydis*
1	> 50	> 50	n.a
2	25	> 50	1,000
3	> 42	-	n.t.
4	> 45	> 45	n.a
Nystatin	-	-	20
Nourseothricin	-	-	2,000

* n.a : not active, n.t. – not tested

Table S3. Cytotoxicity of the isolated compounds **1-4** measured after 24 h of incubation and reported as IC_{50} (μM)

Compound	L5178Y	Ramos	Jurkat J16
1	n.a	n.a	n.a
2	n.a	n.a	n.a
3	1.3	28	20
4	n.a	n.a	n.a
Kahalalide F	4.3	-	-
Staurosporine	-	1.1	-

* n.a : not active

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