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Hymenosetin, a 3-decalinoyltetramic acid antibiotic from cultures of the ash dieback pathogen, *Hymenoscyphus pseudoalbidus*

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Abstract

A 3-decalinoyltetramic acid, for which the trivial name hymenosetin is proposed, was isolated from crude extracts of a virulent strain of the ash dieback pathogen, *Hymenoscyphus pseudoalbidus* (= "*Chalara fraxinea*"). This compound was produced only under certain culture conditions in submerged cultures of the fungus. Its planar structure was determined by NMR spectroscopy and by mass spectrometry. The absolute stereochemistry was assigned by CD spectroscopy and HETLOC data. Hymenosetin exhibited broad spectrum antibacterial and antifungal activities (including strong inhibition of MRSA, as well as moderate cytotoxic effects. So far, the metabolite proved inactive in assays for evaluation of phytotoxicity, whereas viridiol, another secondary metabolite known from *H. pseudoalbidus*, was regarded as phytotoxic principle of the pathogen against its host, *Fraxinus excelsior*. Further studies will show whether hymenosetin constitutes a defence metabolite that is produced by the pathogenic fungus to combat other microbes and fungi in the natural environment.

Keywords

Hymenoscyphus pseudoalbidus, ash dieback, structure elucidation, secondary metabolite, natural products, tetramic acid, MRSA

1. Introduction

In 2010, the fungus *Hymenoscyphus pseudoalbidus* (Helotiales, Ascomycota) was proven to be the causal agent of ash dieback (Queloz et al., 2011, Zhao et al., 2012), a disease that has rapidly spread across Europe in the course of the last 20 years. This notorious pathogen can only be found in ash trees (*Fraxinus*) and is responsible for severe dieback of the European ash

(*Fraxinus excelsior*). *Hymenoscyphus pseudoalbidus* is an invasive species, probably introduced from Japan (Zhao et al., 2012). In its assumed country of origin, *H. pseudoalbidus* is associated with a different species of *Fraxinus* (*F. mandshurica*), in which it causes only mild or no disease symptoms (Zhao et al., 2012). The focus of several research groups is presently directed towards identifying the virulence factors responsible for pathogenicity of *H. pseudoalbidus*, with the subsequent goal of developing targeted strategies to combat the disease[1]. Junker et al. (2013) recently showed that the concentration of the phytotoxin, viridiol (Fig. 1, **1**; Moffatt et al., 1969), which is produced by isolates of both *H. pseudoalbidus* and *H. albidus*, does not correlate with the virulence of the respective strains. Their conclusion was that other, as yet unidentified, virulence factors must be responsible for the pathogenicity of *H. pseudoalbidus.*

To identify these potential virulence factors, we studied three strains of *H. pseudoalbidus* that varied in their virulence (Junker et al., 2013) for their secondary metabolite production in submerged culture. The crude extracts were analysed by HPLC-MS and subjected to a classical antimicrobial screening using fungal and bacterial test organisms in a serial dilution assay. Two extracts of the most virulent strain studied showed extraordinarily strong activity against Gram-positive bacteria and were therefore subjected to bioassay-guided fractionation to determine the active principle. Herein, we report on the isolation and structure elucidation, as well as on the absolute stereochemistry of a novel 3-decalinoyltetramic acid derivative. In addition, biological activity of hymenosetin, including the evaluation of phytotoxicity, is described.

[1] For summary of the current status see Homepage of COST Action FP1103.
http://www.cost.eu/domains_actions/fps/Actions/FP1103

2. Results and Discussion

Screening for antibiotic activities was performed with three strains of *H. pseudoalbidus* that had been selected from a previous study (Junker et al., 2013; Table 1), based on their virulence in several culture media as described in Experimental. Crude extracts obtained from mycelia and culture filtrates of submerged cultures were tested. Whereas the majority of extracts was found devoid of bioactivity (unpublished data), only samples from prolonged fermentations in ZM/2 medium (after 42 days) of strain C492 showed prominent antibacterial effects. These conditions were therefore used for refermentation and scale-up production in order to isolate and identify the active principle. Bioactivity-guided fractionation by RP-HPLC, with *Bacillus subtilis* as indicator organism, led to the isolation of 2.2 mg hymenosetin (**2**; see Fig. 2) as a colourless oil, which constituted the active principle.

The HR-ESI-MS data of hymenosetin (**2**) supported a molecular formula of $C_{23}H_{33}NO_4$ for the molecular ion $[M+H]^+$ (*m/z* 388.2495), implying 8 degrees of unsaturation. The ^1H spectra in CD_3OD, CD_3OH, C_6D_6 and DMSO-d_6 gave abnormally broad signals for multiple resonances, presumably due to tautomerism. However, individual resonances were clearly resolved in the NMR spectra recorded in $CDCl_3$ and thus could easily be assigned for all protons and carbons for the main isomer. The ^1H-NMR and ^{13}C-NMR spectra revealed the presence of five methyls, three methylenes, three olefinic as well as six aliphatic methines, two sp^2 as well as one sp^3 hybridized quaternary carbons and three carbonyls (Table 2). Analysis of the COSY spectrum resulted in the identification of three spin systems (Fig. 3), while HMBC correlations established the planar structure of **2** (Fig. 2). Tetramic acids bearing acyl substituents in the 3-position exist in a mixture of four different tautomers (Royles, 1995). For clarity the classical presentation is chosen in Fig. 2.

Hymenosetin (**2**) is a new member of the group of 3-decalinoyltetramic acids, of which equisetin (**3**) is the first known and best-studied derivative. Apart from different substitution patterns, the stereochemistry varies significantly between members of this compound family. For **2**, the relative stereochemistry of the decalin moiety was assigned by ROESY correlations. No ROESY correlation could be observed between 6-H and 11-H, which is in agreement with a *trans* ring fusion of the six-membered rings. Furthermore, 1,3-diaxial correlations between protons 6-H, 8-H, 10-H_{ax} and between 11-H, 7-H_{ax}, 9-H_{ax} as well as between 12-H_3, 3-H and 6-H indicated a (2*S**,3*R**,6*S**,8*R**,11*R**) relative configuration.

The absolute stereochemistry of **2** was assigned by comparison of its CD characteristics with those of equisetin (**3**), phomasetin (**4**) (Singh et al., 1998) and *epi*-trichosetin (**5**; Inokoshi et al., 2013). The CD spectrum of **2** showed negative cotton effects at 280 nm and 233 nm, consequently a 2*S,5'S* configuration was concluded. Since the very broad signal of the amide proton (δ_H 9.10) showed no NOE effects, the stereochemistry of C-6' could not be established by the traditional method exploiting NOESY correlations in conjunction with $^3J_{H,H}$ coupling constants. The same problem has obviously prevented complete stereochemical characterization of paecilosetin (**6**; Lang et al., 2005), altersetin (**7**; Hellwig et al., 2002) and antibiotic "CJ-17,572" (**8**; Sugie et al., 2002) in earlier studies.

As an alternative strategy to determine the stereochemistry of C-6', we opted for the *J*-based configuration analysis (Matsumori et al., 1999), which utilises $^2J_{C,H}$ and $^3J_{C,H}$ carbon-proton spin coupling constants besides proton-proton spin coupling constants ($^3J_{H,H}$). Primarily, the method was developed for structures containing hydroxyl and alkoxy groups, though the technique was later expanded to nitrogen substituents (Bassarello et al., 2001; Williamson et al., 2004). In acyclic systems such as the side chain of **2**, the configuration of adjacent asymmetric centres can be represented by staggered rotamers (shown in Fig. 4 with the expected magnitude of coupling constants). The small coupling constant of ^2J(H5',C6') = 1.5 Hz indicated an *anti*-like

configuration between proton and hydroxyl function, which is confirmed by small ^{3}J(H5',H6') = 3.4 Hz and small ^{3}J(H5',C7') = 1.4 Hz coupling constants, both indications of *gauche*-like configurations between H5'/H6' and H5'/C7'. The small coupling constant of ^{2}J(H6',C5') = 0.7 Hz indicated an *anti*-like configuration of H6' to the nitrogen atom of the tetramic acid moiety, demonstrating a *R* configuration at C6' and completing the stereo chemical assignment of **2**. The stereochemistry of C5' and C6' is identical with the stereochemistry of C2 and C3 in threonine. As shown for the biosynthesis of equisetin, decalinoyl tetramic acids constitute polyketide-nonribosomal peptide hybrids (Boettger and Hertweck, 2013), which are built up from an octaketide respectively nonaketide and an amino acid. Thus, threonine can be considered as a non-alternated building block of **2**.

Using a standard of hymenosetin (**2**), the crude extracts of the other strains studied were analysed for the presence of the new compound by means of HPLC-MS. Hymenosetin (**2**) was found in minor quantities in the crude extracts (mycelium and supernatant) of strain C444 derived from fermentations in ZM/2 medium, but was not detected in any of the extracts derived from fermentations of strain EL120. This finding agrees with the lack of significant antibacterial effects against Gram-positive bacteria observed with culture extracts of these isolates of *H. pseudoalbidus*.

3-decalinoyltetramic acid antibiotics are widespread in ascomycetes and have often been discovered in natural product screening owing to their extraordinarily strong antimicrobial activity, mostly against Gram-positive bacteria. In addition, many of them showed cytotoxic effects (Schobert & Schlenk, 2008). For some of these compounds, antifungal activity against the yeast, *Candida albicans,* has also been described (Boros et al., 2003; Segeth et al., 2003). However, there is only one record regarding the activity of 3-decalinoyltetramic acids against filamentous fungi (Lang et al., 2005). Furthermore, some derivatives such as equisetin and phomasetin showed additional biological activities in vitro. For instance, inhibition of HIV

integrase was observed for equisetin and phomasetin (Singh et al., 1998), and trichosetin exhibited phytotoxic effects against callus cultures of *Catharanthus roseus* (Marfori et al., 2003). Interestingly, the latter study is also one of the few examples in the literature where elicitation of a fungal secondary metabolite was attained in dual culture with a plant. Phytotoxicity was also detected for equisetin and *epi*-equisetin against seed and seedlings of various dicotyledonous and monocotyledonous plants (Wheeler et al., 1999).

In our study, the antimicrobial effects of hymenosetin (**2**) were assessed in the serial dilution assay against a broad panel of bacteria and fungi (Table 3). The compound showed strong activity against all tested Gram-positive bacteria, including methicillin resistant *Staphylococcus aureus* (MRSA), whereas Gram-negative bacteria were not affected. In addition, non-selective activity was observed against filamentous fungi, while yeast-like fungi varied in their sensitivity. In a cytotoxicity assay against the mouse fibroblast cell line L929, a moderate IC_{50} value (8 µg/ml) was observed for **2**. Furthermore, hymenosetin (**2**) showed no phytotoxic effects against *Fraxinus excelsior* seeds and leaves, nor against seeds of the grass species *Agrostis stolonifera*. In contrast, application of viridiol in leaf segment tests resulted in necroses at the site of application (Andersson et al., 2010). In general, the results of the current study agree with biological effects that were previously reported for other fungal 3-decalinoyltetramic acids.

3. Conclusion

Hymenosetin (**2**) is a new member of the family of 3-decalinoyltetramic acid antibiotics, of which several compounds had previously been described from various genera of filamentous Ascomycota and characterised extensively for their biological effects. Examples are equisetin (a well-known “mycotoxin” from *Fusarium equiseti*; Burmeister et al., 1974), phomasetin (from *Fusarium heterosporum* and a *Phoma* sp.; Singh et al., 1998), altersetin (from endophytic

Alternaria spp.; Hellwig et al., 2002) and trichosetin (from *Trichoderma harzianum*; Marfori et al., 2002).The latter compound was even reported to possess significant phytotoxic effects (Marfori et al., 2003), whereas all other derivatives of this type are known to have broad spectrum antimicrobial and cytotoxic effects. As hymenosetin (**2**) has thus far only been obtained from laboratory cultures, its role in the pathogenesis of ash dieback remains unclear. The fact that production rates of this new compound in the three tested strains correlate with virulence of the isolates (Junker et al., 2013), suggests that the metabolite might constitute an important, hitherto unknown, pathogenicity factor. In that case, the tests for phytotoxicity used were not the correct ones to the function of hymenosetin (**2**) *in vivo*. On the other hand, this secondary metabolite may not act as a virulence factor directed against the host *in planta*, but could rather constitute a defence compound that the pathogen produces to combat competing microorganisms and fungi. Further studies, e.g. to compare the production of (**2**) in additional virulent and avirulent strains of *H. pseudoalbidus* and in the avirulent sister species, *H. albidus,* as well as with its production in dual culture with fungal endophytes isolated from apparently resistant ash trees, are currently under way in order to further elucidate the significance of the new compound.

4. Experimental

4.1. General

Optical rotations were determined with a Perkin-Elmer 241 spectrometer; IR spectra were measured with a Spectrum 100 FTIR spectrometer (Perkin Elmer), UV spectra were recorded with a Shimadzu UV-Vis spectrophotometer UV-2450, CD spectra were recorded on a JASCO spectropolarimeter, model J-815. NMR spectra were recorded with Bruker Ascend 700 spectrometer with a 5 mm TXI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) and Bruker AV II-600 (^{1}H

600 MHz, ^{13}C 150 MHz) spectrometers. HR-ESI-MS mass spectra were obtained with an Agilent 1200 series HPLC-UV system [column 2.1×50 mm, 1.7 μm, C_{18} Acquity UPLC BEH (Waters), solvent A: H_2O + 0.1 % formic acid; solvent B: AcCN + 0.1 % formic acid, gradient: 5 % B for 0.5 min increasing to 100 % B in 19.5 min, maintaining 100 % B for 5 min, flow rate 0.6 ml x min^{-1}, UV/Vis detection 200-600 nm] combined with an ESI-TOF-MS (Maxis, Bruker) [scan range 100 – 2500 *m/z*, rate 2 Hz, capillary voltage 4500 V, dry temperature 200 °C].

4.2. Fungal material

The three strains of *Hymenoscyphus pseudoalbidus* used in this study (designation EL120, C444 and C492) had been isolated from necrotic stem lesions of *Fraxinus excelsior* from different locations in northern Germany. The origin of these three strains as well as their rDNA ITS GenBank accession numbers are listed in Table 1. This information as well as the viridiol concentration of their culture extracts, the activity of these culture extracts in tests for virulence, and the virulence of the isolates after inoculation into seedlings of *F. excelsior* were previously reported by Junker et al. (2013).

4.3. Cultivation, extraction and isolation

4.3.1. Cultivation

Hymenoscyphus pseudoalbidus EL120, C444 and C492 were cultivated in three different liquid media (*YMG medium*: 1.0 % malt extract, 0.4 % glucose, 0.4 % yeast extract, pH 6.3; *Q6/2 medium*: 1.0 % glycerol, 0.25 % glucose, 0.5 % cotton seed flour, pH 7.2; *ZM/2 medium*: 0.5 % molasses, 0.5 % oatmeal, 0.15 % glucose, 0.4 % sucrose, 0.4 % mannitol, 0.05 % edamine, 0.05 % ammonium sulphate, 0.15 % calcium carbonate, pH 7.2) in Erlenmeyer flasks (500 ml)

filled each with 200 ml media. These media were chosen because previous studies (Stadler et al., 2003; Bitzer et al., 2008) had revealed that they are optimal for attaining complementary secondary metabolite profiles of filamentous ascomycetes.

The submerged cultures were incubated at 23°C in the dark on a rotary shaker at 140 rpm. To obtain hymenosetin, it seems crucial to continue fermentation until after the carbon source has been depleted for a substantial time. Fermentation of strain C492, which produced **2** in higher concentrations in ZM/2 medium (in contrast to EL120 and C444), was terminated only after 42 days.

4.3.2. Extraction and isolation of hymenosetin (**2**)

Hymenosetin was extracted from cultures of strain C492 in ZM/2 medium using the following procedure: The mycelium was separated from the culture fluid via vacuum filtration and extracted twice with acetone (200 ml) in an ultrasonic bath for 30 min. The extracts were filtered, combined and concentrated by means of a rotary evaporator. The remaining aqueous phase was subjected to a solvent extraction with water : ethyl acetate (50 ml : 50 ml). Subsequently the organic phase was filtered over anhydrous sodium sulphate and evaporated. 30 mg crude extract was obtained from the mycelium filtered from 200 ml culture in ZM/2 medium. The culture filtrate was extracted twice with ethyl acetate (200 ml) in an ultrasonic bath for 30 min. The organic phases were combined, filtered with sodium sulfate (anhydrous) and then ethyl acetate was removed in a rotary evaporator, yielding 14 mg crude extract from the culture filtrate. Since HPLC-MS analysis revealed a highly similar composition of the extracts from mycelia and supernatant, they were united. The complete crude extract was adsorbed onto a RP-18 cartridge and eluted with methanol. By using a preparative RP HPLC (column: VP Nucleodur C18 ec, 250 x 21 mm, 5 µm, Macherey-Nagel; gradient: 60 % to 100 % acetonitrile with 0.5% acetic acid in 30 min, 100 % for 15 min; flow rate: 15 ml/min), the filtered raw extract

was fractionized into 90 fractions. Fraction 52/53 (eluted 26.0-27.5 min) contained 2.2 mg of the novel active compound **2**; its purity was confirmed by high resolution HPLC-MS (R_t = 23.0-24.2 min).

4.3.3. Characteristics of hymenosetin (**2**)

Yellow oil, $[\alpha]^{25}_D$ -748 (*c* 0.1, CH_2Cl_2); CD (MeOH) λ_{max} nm ($\delta\varepsilon$): 233 nm (-8.3), 253 nm (-5.1), 280 nm (-14.4); UV (MeOH) λ_{max} (log ε) 228.8 nm (sh), 252.6 nm (4.61) 287.6 (4.89); IR (KBr) 3401, 2918, 2852, 1660, 1583, 1452, 1376, 1132, 969, 620 cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$, TMS), ^{13}C-NMR (150 MHz, $CDCl_3$) see Table 2; HR-ESI-MS *m/z* 388.2479 $[M+H]^+$ (calcd for $C_{23}H_{33}NO_4$, 388.2482).

4.4. Serial dilution assay

Minimum inhibitory concentrations (MIC) were determined in a serial dilution assay carried out in a similar manner as previously described (Okanya et al., 2011; Surup et al., 2013) using various test organisms for antibacterial and antifungal activities (Table 3). The assays were conducted in 96-well microtiter plates in EBS-medium (0.5 % casein peptone, 0.5 % glucose, 0.1 % meat extract, 0.1 % yeast extract, 50mM HEPES [11.9 g/L], pH 7.0) for bacteria and MYC-medium (Okanya et al., 2011) for yeasts and filamentous fungi. Depending on the activity, the starting concentration of **2** as well as of the reference drugs varied from 67 to 6.7 µg/ml.

4.5. Cytotoxicity assay

The *in vitro* cytotoxicity assay with the established mouse fibroblast cell line L929 was performed as reported by Okanya et al. (2011). The IC_{50} value of hymenosetin (**2**) was determined to be 8 µg/ml.

4.6. Phytotoxicity assays

For evaluation of its phytotoxicity, hymenosetin (**2**) was tested in germination assays using seeds of *Fraxinus excelsior* and of the grass *Agrostis stolonifera,* as well as in a leaf segment test with healthy leaves of *F. excelsior* (slightly modified procedure of Junker et al., 2013). Instead of using 25 µl culture extract (40mg/ml), 10 µL aliquots of the pure compound **2** (1 mM dissolved in 100 % EtOH) were applied to individual leaves. The test was run once with ten parallels. The germination test with *F. excelsior* seeds followed the method of Junker et al. (2013), with the exception that 500 µl of **2** (at 1 mM solved in 100 % EtOH) were added to H_{10} medium before solidification occurred. This experiment was conducted once with five parallels (6 seeds/ Petri dish). The seedling germination test with *A. stolonifera* was performed in a similar manner as previously reported (Schulz et al., 2008). However, deviating from the protocol, 10 µl of **2** (1 mM) dissolved in 100 % EtOH was added to 240 µl Gamborg B5 Medium (Sigma-Aldrich) with 30 seeds/well in a 96-well microtiter plate. Likewise, wells with only Gamborg B5 Medium and 30 seeds were applied as negative control. The germination test with *A. stolonifera* was run in triplicate. The 96-well plate was incubated for two weeks in a growth chamber at 20°C under artificial light (8 h; 800 x 10 Lux).

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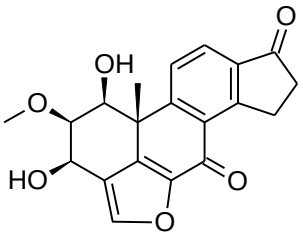
Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/xxx



1

Fig. 1. Chemical structure of the phytotoxin viridiol (**1**; Moffatt et al., 1969).

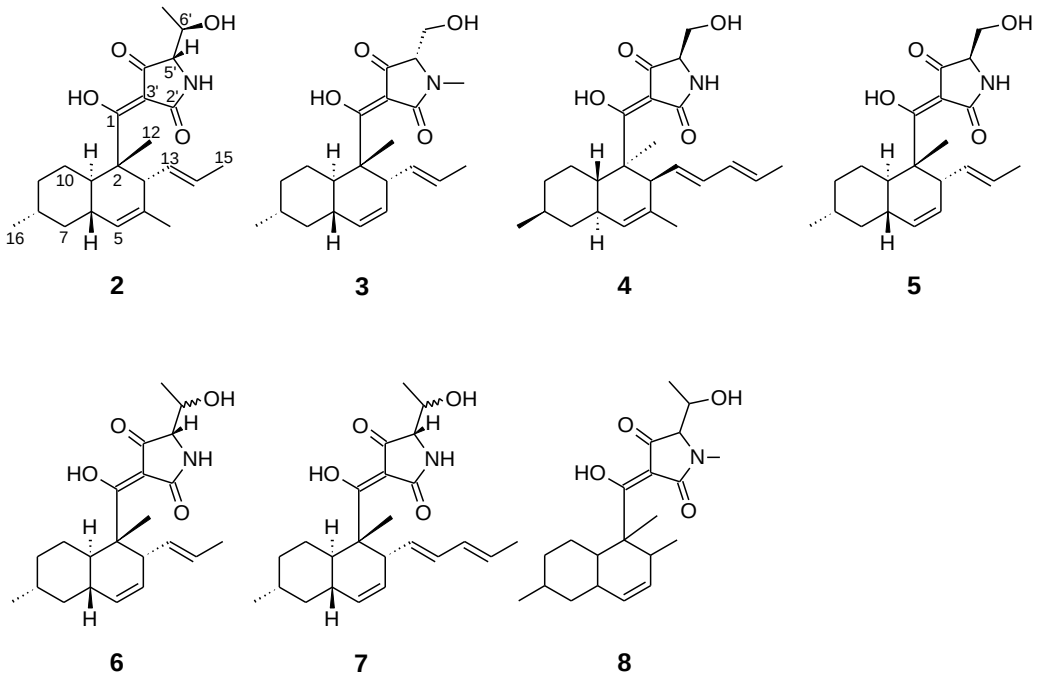


Fig. 2. Chemical structures of the new compound hymenosetin (**2**) from *H. pseudoalbidus* and of related 3-decalinoyltetramic acid antibiotics, equisetin (**3**), phomasetin (**4**) (Singh et al., 1998), *epi*-trichosetin (**5**; Inokoshi et al., 2013), paecilosetin (**6**; Lang et al., 2005), altersetin (**7**; Hellwig et al., 2002) and “Antibiotic CJ-17,572” (**8**; Sugie et al., 2002).

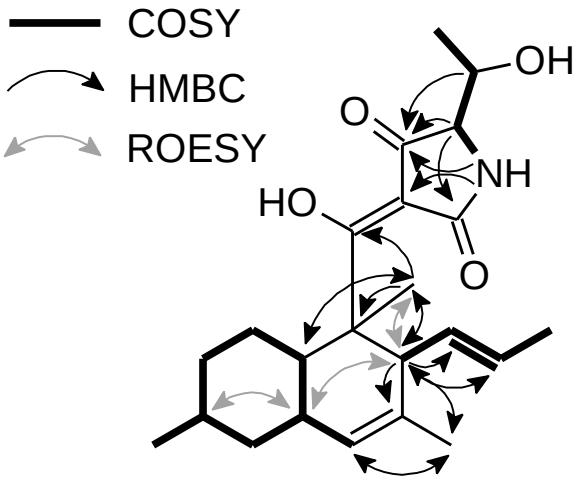


Fig. 3. Selected COSY, HMBC and ROESY correlations for hymenosetin (**2**).

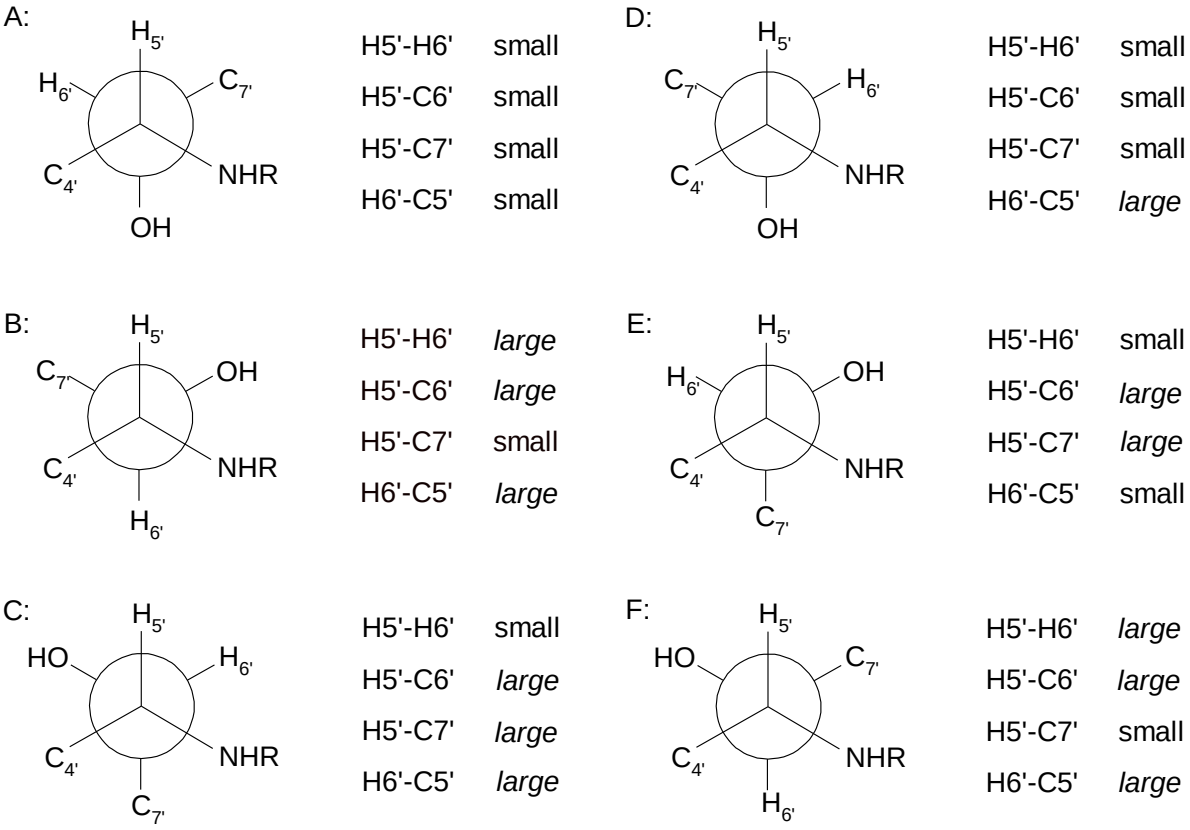


Fig. 4. *J*-based analysis of six hypothetical rotamers with 5'*S*,6'*R* (A – C) and 5'S,6'S (D – F) configuration to determine the stereochemistry of hymenosetin (**2**). Expected couplings contrary (shown in *italics*) to the observed ones (^{3}J(H5',H6') = 3.2 Hz; ^{2}J(H5',C6') = 1.5 Hz; ^{3}J(H5',C7') = 1.4 Hz; ^{2}J(H6',C5') = 0.7 Hz) exclude all configurations except A.

Table 1. Origin of the *H. pseudoalbidus* strains as well as their rDNA ITS GenBank accession numbers and their DSM accession numbers.

Strain no.	Location and year	Identified by	rDNA ITS GenBank accession no.	DSM no.
EL120	Elm forest near Erkerode (Lower Saxony, Germany), necrotic tissue from *Fraxinus excelsior*, 2009	S. Draeger	KC576539	DSM 28189
C444	Tree nursery, Nauen (Brandenburg, Germany), necrotic tissue from *F. excelsior*, 2009	J. Schumacher	KC576533	DSM 28190
C492	Tree nursery, Ellerhoop (Schleswig-Holstein, Germany), necrotic tissue from 3-year-old *F. excelsior* seedling, 2008	J. Schumacher	KC576536	DSM 28191

Table 2. NMR data (^{1}H 600 MHz, ^{13}C 150 MHz) of hymenosetin (**2**) in chloroform-*d*.

Position	δC, type	δH, m (J in Hz)
1	200.5, C	
2	49.5, C	
3	49.4, CH	3.10, brd (9.6)
4	132.0, C	
5	125.6, CH	5.16, brs
6	39.1, CH	1.83, m
7	42.5, CH_2	1.80, m
		0.88, m
8	33.5, CH	1.52, m
9	35.8, CH_2	1.78, m
		1.12, m
10	28.2, CH_2	1.96, m
		1.05, m
11	39.8, CH	1.68, m
12	13.6, CH_3	1.41, brs
13	130.6, CH	5.14, m
14	127.7, CH	5.26, m
15	17.9, CH_3	1.56, d (6.6)
16	22.2, CH_3	1.60, brs

17	22.5, CH_3	0.92, d (6.4)
2’	179.4, C	
3’	100.3, C	
4’	190.9, C	
5’	65.3, CH	3.70, d (4.9)
6’	67.8, CH	4.05, m
7’	19.5, CH_3	1.32, d (6.4)
NH		6.05, brs

Table 3. Minimal inhibitory concentrations (MIC) of hymenosetin (**2**) in liquid medium. MIC values are given in µg/ml. (/) indicates lack of activity. Concentration of tested bacteria and yeasts: OD_{600} =0.01 (8 x 10^6 cell/ml).

Test organisms	**MIC [µg/ml]**	
	Hymenosetin (2)	**Reference [a,b,c,d]**
Gram-positive bacteria		
Micrococcus luteus DSM 20030	1.0	2.1 [a]
Mycobacterium diernhoferi DSM 43524	1.0	≤ 0.25 [a]
Nocardia sp. DSM 43069	1.0	0.52 [a]
Nocardioides simplex DSM 20130	0.52	16.6 [a]
Paenibacillus polymyxa DSM 36	4.2	6.7 [a]
Staphylococcus aureus DSM 346	0.52	≤ 0.25 [a]
methicillin-resistant *S. aureus* (MRSA) strain N315[2]	0.83	0.52 [b]
Gram-negative bacteria		
Chromobacterium violaceum DSM 30191	/	1.0 [a]
Escherichia coli DSM 1116	/	1.0 [a]
Pseudomonas aeruginosa DSM 50071	/	67 [c]
Yeasts		
Candida albicans DSM 1665	/	4.2 [d]
Nematospora coryli DSM 6981	1.0	3.3 [d]
Pichia anomala DSM 6766	/	4.2 [d]

[2] Strain has been deposited at DSMZ, Braunschweig, Germany, but no accession number was obtained as yet.

Rhodotorula glutinis DSM 10134	16.6	0.52 [d]
Schizosaccharomyces pombe DSM 70572	/	8.3 [d]
Trichosporon oleaginosus DSM 11815	33.3	1.0 [d]
Filamentous fungi		
Aspergillus clavatus DSM 816	33.3	2.1 [d]
Hormoconis resinae DSM 1203	4.2	0.25 [d]
Mucor hiemalis DSM 2656	4.2	2.1 [d]
Penicillium capsulatum DSM 2210	33.3	16.6 [d]

[a] oxytetracycline hydrochloride (1 mg/ml); [b] vancomycin (5 mg/ml); [c] gentamycin (10 mg/ml); [d] nystatin (1 mg/ml).

References

Andersson, P.F., Johansson, S.B.K., Stenlid, J., Broberg, A., 2010. Isolation, identification and necrotic activity of viridiol from *Chalara fraxinea*, the fungus responsible for dieback of ash. Forest Pathol. 40, 43–46.

Bassarello, C., Bifulco, G., Evidente, A., Riccio, R., Gomez-Paloma, L., 2001. Stereochemical studies on ascaulitoxin: a *J*-based NMR configurational analysis of a nitrogen substituted system. Tetrahedron Lett. 42, 8611–8613.

Bitzer, J., Læssøe, T., Fournier, J., Kummer, V., Decock, C., Tichy, H.-V., Piepenbring, M., Peršoh, D., Stadler, M., 2008. Affinities of *Phylacia* and the daldinoid Xylariaceae, inferred from chemotypes of cultures and ribosomal DNA sequences. Mycol. Res. 112, 251–270.

Boros, C., Dix, A., Katz, B., Vasina, Y., Pearce, C., 2003. Isolation and identification of Cissetin-a setin-like antibiotic with a Novel cis-octalin ring fusion. J. Antibiot. 56, 862–865.

Boettger, D., Hertweck, C., 2013. Molecular diversity sculpted by fungal PKS-NRPS hybrids. ChemBioChem 14, 28–42.

Burmeister, H.R., Bennet, G.A., Vesonder, R.F., Hesseltine, C.W., 1974. Antibiotic produced by *Fusarium equiseti* NRRL 5537. Antimicrob. Agents Chemother. 5, 634–639.

Hellwig, V., Grothe, T., Mayer-Bartschmid, A., Endermann, R., Geschke, F.R., Henkel, T., Stadler, M., 2002. Altersetin, a new antibiotic from cultures of endophytic *Alternaria* spp. J. Antibiot. 55, 881–892.

Inokoshi, J., Shigeta, N., Fukuda, T., Uchida, R., Nonaka, K., Masuma, R., Tomoda, H., 2013. *Epi*-trichosetin, a new undecaprenyl pyrophosphate synthase inhibitor, produced by *Fusarium oxysporum* FKI-4553. J. Antibiot. 66, 1–6.

Junker, C., Mandey, F., Pais, A., Ebel, R., Schulz, B., 2013. *Hymenoscyphus pseudoalbidus* and *Hymenoscyphus albidus*: viridiol concentration and virulence do not correlate. Forest Pathol., doi: 10.1111/efp.12066.

Lang, G., Blunt, J.W., Cummings, N.J., Cole, A.L.J., Munro, M.H.G., 2005. Paecilosetin, a new bioactive fungal metabolite from a New Zealand isolate of *Paecilomyces farinosus*. J. Nat. Prod. 68, 810–811.

Marfori, E.C., Kajiyama, S., Fukusaki, E., Kobayashi, A., 2003. Phytotoxicity of the tetramic acid metabolite trichosetin. Phytochemistry 62, 715–721.

Marfori, E.C., Kajiyama, S., Fukusaki, E., Kobayashi, A., 2002. Trichosetin, a novel tetramic acid antibiotic produced in dual culture of *Trichoderma harzianum* and *Catharanthus roseus* callus. Z. Naturforsch. 57c, 465–470.

Matsumori, N., Kaneno, D., Murata, M., Nakamura, H., Tachibana, K., 1999. Stereochemical determination of acyclic structures based on carbon-proton spin-coupling constants. A method of configuration analysis for natural products. J. Org. Chem. 64, 866–876.

Moffatt, J.S., Bu´Lock, J. D., Yuen, T.H., 1969. Viridiol, a steroid-like product from *Trichoderma viride*. J. Chem. Soc., Chem. Commun. 839.

Okanya, P.W., Mohr, K.I., Gerth, K., Jansen, R., Müller, R., 2011. Marinoquinolines A – F, pyrroloquinolines from *Ohtaekwangia kribbensis* (Bacteroidetes). J. Nat. Prod. 74, 603–608.

Queloz, V., Grünig, C.R., Berndt, R., Kowalski, T., Sieber, T.N., Holdenrieder, O., 2011. Cryptic speciation in *Hymenoscyphus albidus*. Forest Pathol. 41, 133–142.

Royles, B.J.L., 1995. Naturally occurring tetramic acids: Structure, isolation, and synthesis. Chem. Rev. 95, 1981–2001.

Schobert, R., Schlenk, A., 2008. Tetramic and tetronic acids: An update on new derivatives and biological aspects. Bioorg. Med. Chem. 16, 4203–4221.

Schulz, B., Draeger, S., Dela Cruz, T.E., Rheinheimer, J., Siems, K., Loesgen, S., Bitzer, J., Schlörke, O., Zeeck, A., Kock, I., Hussain, H., Dai, J., Krohn, K., 2008. Screening strategies for obtaining novel, biologically active, fungal secondary metabolites from marine habitats. Bot. Mar. 51, 219–234.

Segeth, M.P., Bonnefoy, A., Brönstrup, M. Knauf, M., Schummer, D., Toti, L., Vértesy. L. Wetzel-Raynal, M.-C., Wink, J., Seibert, G., 2003. Coniosetin, a novel tetramic acid antibiotic from *Coniochaeta ellipsoidea* DSM 13856. J. Antibiot. 56, 114–122.

Stadler, M., Tichy, H.-V., Katsiou, E., Hellwig, V., 2003. Chemotaxonomy of *Pochonia* and other conidial fungi with *Verticillium*-like anamorphs. Mycol. Progr. 2, 95–122.

Singh, S.B., Zink, D.L., Goetz, M.A., Dombrowski, A.W., Polishook, J.D., Hazuda, D.J., 1998. Equisetin and a novel opposite stereochemical homolog phomasetin, Two fungal metabolites as inhibitors of HIV integrase. Tetrahedron Lett. 39, 2243–2246.

Sugie, Y., Dekker, K.A., Inagaki, T., Kim, Y.J., Sakakibara, T., Sakemi, S., Sugiura, A., Brennan, L., Duignan, J., Sutcliffe, J.A., Kojima, Y., 2002. A novel antibiotic CJ-17,572 from a fungus, *Pezicula* sp.. J. Antibiot. 55, 19–24.

Surup, F., Mohr, K.I., Jansen, R., Stadler, M., 2013. Cohaerins G - K, azaphilone pigments from *Annulohypoxylon cohaerens* and absolute stereochemistry of cohaerins C – K. Phytochemistry 95, 252–258.

Wheeler, M.H., Stipanovic, R.D., Puckhaber, L.S., 1999. Phytotoxicity of equisetin and *epi*-equisetin isolated from *Fusarium equiseti* and *F. pallidoroseum.* Mycol. Res. 1003, 967–973.

Williamson, R.T., Singh, I.P., Gerwick, W.H., 2004. Taveuniamides: new chlorinated toxins from a mixed assemblage of marine cyanobacteria. Tetrahedron 60, 7025–7033.

Zhao, Y.J., Hosoya, T., Baral, H.O., Hosaka, K., Kakishima, M., 2012. *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. Mycotaxon 122, 25–41.