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Graphical Abstract

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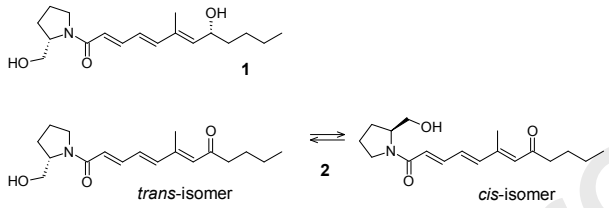

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Viriditins from *Byssochlamys spectabilis*, their stereochemistry and biosynthesis

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ABSTRACT

Byssochlamys spectabilis (anamorph *Paecilomyces variotii*) strain 10536 was isolated as an endophyte from grapevine and investigated for its secondary metabolite production. Cultures of *B. spectabilis* yielded the known compound viriditin A (**1**) and its new derivative viriditin B (**2**), which showed pronounced *cis-trans*-amid isomerism. The so far unknown absolute configuration of C–2 and C–13 in **1** were assigned by Mosher's method. Marfey's method confirmed 2*S* stereochemistry after Jones oxidation and hydrolysis. A series of feeding experiments with [1-^{13}C], [2-^{13}C] and [1, 2-$^{13}C_2$]-acetate as well as [methyl-^{13}C]-methionine indicated a polyketide biosynthetic pathway. Compound **1** showed weak cytotoxicity against the cell line KB3.1 with an IC_{50} = 30µg/ml.

Grapevine (*Vitis vinifera* L) is a native European plant that has been used for wine production since ancient times.[1] Like many other plants, grapevine can be asymptomatically colonized by microorganisms, which are called endophytes.[2] It has been established that endophytes interact with grapevine metabolism when colonizing their roots and can both activate and block pathways for plant defense, which suggests that *in planta*, a chemical exchange between endophytes and the host takes place. Additionally, endophytes must co-habit with many organisms that are competitors for space and nutrients. This species co-occurrence and resource overlap forces interactions that can result in the production of secondary metabolites.[3,4]

Endophytic fungi also live closely associated with wild and cultivated grapevine. For example, in *V. vinifera* fungal endophytic diversity varies according to host cultivar type showing the tight association of fungal endophytes with their plant host.[5]

The genus *Byssochlamys* (Thermoascaceae: Eurotiales) consists of fungal species closely related to the genera *Penicillium* and *Aspergillus*.[6] It forms a well-defined clade in the *Eurotiales*.[7]

Members of *Byssochlamys* are typically isolated from the soil, and thus fruit near the plant can be contaminated with conidia from the fungus.[8] Some species of *Byssochlamys* produce highly resistant ascospores that are both heat and low oxygen tension resistant,[9] which pose a great challenge for disinfection protocols based on pasteurization. However treatment with chemical agents shows little recovery of either dormant and heat- activated spores in the anamorphic phase of *B. spectabilis* (including disinfection with solutions at 200 and 500 ppm of chlorine dioxide and citric acid-activated sodium chlorite).[10]

Also, species of the genus have been found in a remarkable number of marines sources, including the white mangrove *Laguncularia racemosa* and marine algae showing their ability of adaptation to extreme environments probably explained by their rather strong spore adaptation.[11]

Since endophytes have great potential for natural product synthesis,[12] we investigated the secondary metabolite production of the endophytic fungus *Byssochlamys spectabilis* strain 10536 (DSM 109444) after its isolation from grapevine (Fig. S1 and S2). In the crude extracts from cultures of strain 10536, we observed three main peaks (Fig. S3).

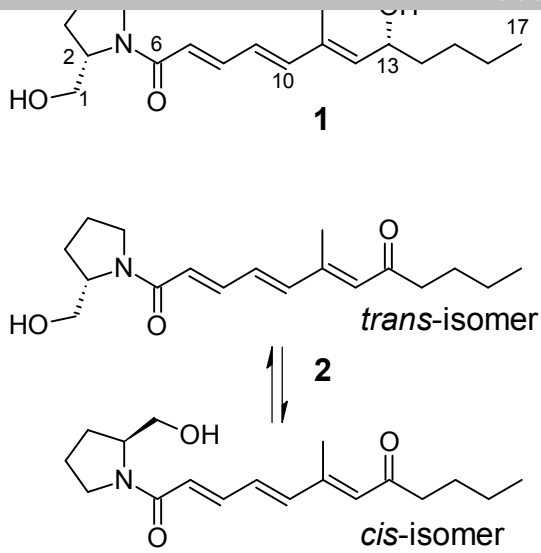
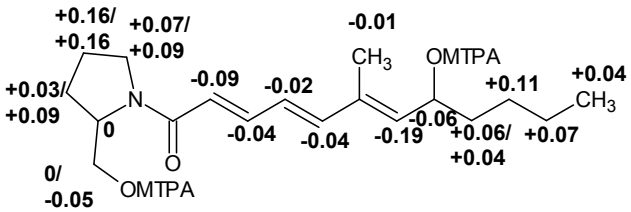


Figure 1. Structure of viriditin A (**1**) and its new derivative viriditin B (**2**), which showed a pronounced *cis*-*trans*-amide isomerism.

Viriditin (**1**) was isolated by preparative HPLC as a pale-green oil, with the molecular formula $C_{18}H_{29}NO_3$ determined by HRESIMS and indicative of five units of unsaturation. The proton and ^{1}H,^{13}C HSQC spectra showed signals of two methyls, five olefinic and two aliphatic methines (one of them an oxymethine) along with seven methylenes (one of them an oxymethylene). In addition, the carbon spectrum revealed the presence of one conjugated ester and one quaternary olefinic carbon. Detailed analysis of the COSY, TOCSY and HMBC data confirmed its planar structure and confirmed its identity as viriditin (Fig. 1).[13] Its structure, although partially elucidated, had not been completely resolved in terms of stereochemistry. Since we have provided evidence of a new derivative of **1**, we propose to rename this compound as viriditin A. Also because no stereochemistry had been assigned to the C–2 and C–13 stereocenters of **1**, the absolute configuration of C–13 was assigned by Mosher´s method.[14] Because $\Delta\delta^{SR}$ values of α-methoxy-α-(trifluoro-methyl-) phenylacetic acid (MTPA) esters were positive for 14–H_2 – 17–H_3 and negative for 1–H_2 – 12–H (Fig. 2), a 13*R* configuration was deduced. Furthermore, the signal patterns and chemical shift differences of the germinal protons 1–H_a and 1–H_b ($\Delta\delta_H$ +0.25 ppm for the (*S*)-MPTA ester, $\Delta\delta_H$ +0.20 ppm for the (*R*)-MPTA ester) indicated a 2*S* configuration. To confirm the stereochemistry of C–2, **1** was oxidized and hydrolyzed to yield free proline, which was converted into its Marfey's derivative by treatment with 1-fluoro-2,4-dinitrophenyl-5-L—alanine amide (FDAA).[15] HPLC analysis of the FDAA derivative of the viriditin hydrolysate and those of authentic *S*- and *R*-proline disclosed the presence of *S*-proline in the hydrolysate. Thus, the absolute configuration of **1** was determined as 2*S*,13*R*.

Figure 2. $\Delta\delta^{SR}$ values for MPTA esters of **1** diagnostic for 2*S*,13*R* configuration.



lipophilic fraction that contained the two peaks **2t** and **2c** (Fig. S3), which interconverted into each other after isolation by preparative HPLC. Both **2t** and **2c** had the molecular formula $C_{18}H_{27}NO_3$, as determined by HRESIMS, indicating the formal loss of 2*H* compared to viriditin A (**1**). The proton and carbon spectra of **2t/c** were similar to **1**, with the key differences being the substitution of methine C–13 by a ketone. Consequently, **2** was assigned as the 13-keto derivative of **1** and named viriditin B. However, two sets of resonances were observed in the ^{1}H and ^{13}C spectra for the CH_2–1 to CH–12 part with a ratio of 4:6, indicating indicated spontaneous inter-conversion between *cis*- and *trans*-isomers. The coalescence temperature is at approcimately 358K (Fig. 3). A ROESY correlation between 7–H and 5–H_2 indicated a *trans*-amide configuration of the main isomer, whereas a ROESY correlation was observed between 7–H and 2–H for the minor *cis*–isomer. Although the *cis/trans* isomerization of proline moieties plays a key role in protein folding,[16] it has only rarely been observed for secondary metabolites.[17] A minor isomer can also be observed in the case of **1**. However, less than 5% of **1** exists in the *cis*-amide configuration.

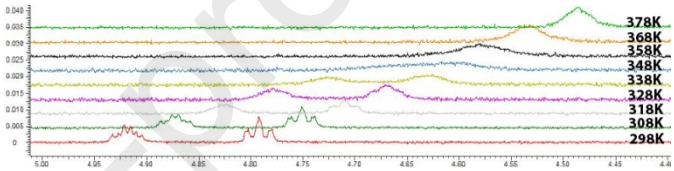


Figure 3. Section of the ^{1}H NMR spectra (700 MHz, DMSO-d_6) of **2** measured at 298 – 378 K showing the coalescence of the 2–H signal for *cis* and *trans*-amid-isomers.

Analysis of the bioactivity revealed that **1** possesses very weak activity against *B. subtilis* (MIC = 300 µg/mL), no activity against any other bacterium of our compilation of test organisms.[18] Additionally, no activity was observed against endophytic gram positive and gram negative bacteria isolated from *V. vinifera* (Table S1). Derivative **2** had no biological activity against the bacterial strains tested. Cytotoxicity was determined against cervical cancer cell line KB-3-1 and mouse fibroblast cell line L929. Only **1** was toxic to KB3.1 with an IC_{50} = 30µg/ml, but no activity was detected against the cell line L929 (Table S2).

To determine the biosynthetic pathway of **1** and **2**, a series of feeding experiments were carried out with stable isotope-labeled precursors. [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$]-acetate as well as [methyl-^{13}C]-methionine. The isotopes were fed to growing cultures of *B. spectabilis* 10536. The incorporation of non-labeled control substances, including L-proline, into the growth medium, did not affect growth of the fungus (Fig. S5A); however it did affect biosynthesis of **1** and **2**. Acetate increased the production of both molecules, but addition of 0.1 mg of L-proline reduced the production of **1** and **2** by half, suggesting a possible anabolic repression of its synthesis. Addition of L-methionine also exerted a negative effect on the biosynthesis of **2**. Analysis of *B. spectabilis* fermentation broths through HPLC-MS demonstrated accumulation of both **1** and **2** (Fig. S5B); production of **1** and **2** reached a concentration peak at 96 h for **1** and 290 h for **2** (Fig. S5C). Both compounds were best produced in a glucose-rich medium compared to other media, even in biomimetic conditions where yeast and even grapevine leaves were added (Fig. S5D ANOVA P value = 0.00004).

spectroscopy.[19] High incorporation rates at positions C–7, C–9, C–11, C–13, C–15 and C–17 of the carbon skeleton upon feeding with [1-^{13}C]-acetate as well as at positions C–6, C–8, C–10, C–12, C–14 and C–16 upon feeding with [2-^{13}C]-acetate clearly indicated a polyketide biosynthetic pathway (Fig. 4, Table 1). Furthermore, a feeding experiment with [1,2-$^{13}C_2$]-acetate proved intact incorporation of six acetate units by strong spin-spin couplings and gave evidence for the direction of the polyketide chain. A feeding experiment with [*methyl*-^{13}C]-methionine showed strong signal enrichment of C–18, demonstrating an S-adenosylmethionin (SAM) mediated methylation. The C–1 – C–5 moiety most likely derives from proline, which has yet to be proved experimentally. The chemical structure, together with the feeding experiments, provides a biosynthesis model for the viriditins. Presumably, biosynthesis of the viriditins starts with the assembly of six acetate units by a polyketide synthase; the fourth unit is methylated with SAM by a methyltransferase.

Table 1. ^{13}C NMR data from feeding experiments on the biosynthetic pathway of **1**

Atom #	δ_c [ppm]	[1-^{13}C-] acetate [a]	[2-^{13}C-] acetate [a]	[1,2-$^{13}C_2$-] acetate [b]	[*methyl*-^{13}C-] methionine [a]
1	67.7	0	0.0	n.o.	0.0
2	61.5	-0.10	0.2	n.o.	0.0
3	28.3	-0.06	-0.01	n.o.	0.0
4	24.4	-0.06	0.9	34	0.1
5	48.1	0.3	-0.7	34	0.05
6	167.6	**1.1**	-0.2	67	n.o.
7	120.9	n.o.	**7.8**	67	0.1
8	143.4	**0.8**	-0.4	56	0.06
9	126.1	n.o.	**6.5**	56	n.o.
10	144.4	**1.4**	-0.3	54	0.1
11	134.8	n.o.	**7.4**	54	n.o.
12	138.9	**1.8**	1.6	48	0.7
13	68.6	n.o.	**8.4**	48	0.02
14	37.2	0.5	-0.3	35	0.1
15	27.5	-0.1	**5.6**	35	0.01
16	22.6	**0.7**	-0.7	35	0.1
17	14.0	-0.03	**6.0**	35	0.2
18	12.8	-0.1	-0.6	n.o.	**19.7**

[a]Values of percentage of incorporation (%I) were normalized to the unlabeled C_1 in the proline moiety.

[b] Coupling constants (in Hz).

n.o. = not observed

Taking into account the common NRPS-PKS hybrid biosynthesis, a proline building block is most likely condensed to a PKS-chain by a non-ribosomal peptide synthase and cleaved off reductively (Fig. 4). Our results point to a textbook style hybrid PKS-NRPS biosynthesis for the viriditins, which resembles the biosynthesis proposed for variotin based on labeling experiments with ^{14}C-labeled precursors[20] The same arrangement, the loading of a PKS chain onto proline building block catalyzed by a NRPS module, most likely also occurs for the closely related structures of scalusamides A–C and formosusins A–C.[21,22]

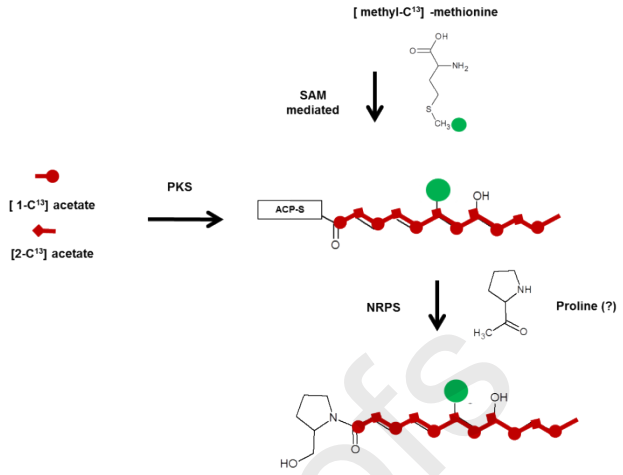


Figure 4. Hypothetical biosynthetic pathway of viriditins. ACP-S= acyl moiety bound to the Acyl Carrier Protein through a sulfur bond. NRPS = Non ribosomal peptide synthase; PKS = Polyketide synthase. Proline = incorporation remains to be experimentally confirmed.

Byssochlamys spectabilis, or rather its anamorph *Paecilomyces variotii,* has been shown producing a great versatility of secondary metabolites with varied bioactivities.[23] These include the antifungals varioxepine A and the pentacyclic triterpenoid betulin,[24,25] the lipopeptide antibiotics leucinostatins,[26] anthraquinones paeciloquinones,[27] the pyridone alkaloid paecilomide[28] and paecilaminol[29]. Cornexistin for example, has a selective herbicide activity against invading mono- a dycotiledoneous plants with no effect in corn, and was proposed as a plant protection strategy[30] whereas variotin[31], isolated from the anamorph *P. variotii* has a strong antimycotic activity against foot skin infections caused by *Tricophyton*, *Dermatophyton*, *Microsporia* and *Dermatomyces* species. Viriditin A (**1**), also an antimycotic, was first isolated from cultures of by *Apergillus viridi-nutans*, together with variotin.[13]

Recently the role of *B. spectabilis* viriditoxin in the antagonism against the plant pathogens *Fusarium moliniforme*, *Biscogniauxia mediterranea* and *Phytophtora cinnamomi* was demonstrated.[32] These are produced by different strains of *Bysssochlamys* although not necessarily by endophytic forms.

With all these metabolites being produced with little nutritional requirements and the fact that *B. spectabilis* can both contaminate man made products and be persistent in them, an immense challenge in terms of sterilization of the good for human consumption is being faced. Strict guidelines show that the *Eurotiales* are a major concern for good manufacturing practices and the metabolites associated with the fungus have to be taken into account when preparing quality control schemes a critical point analysis.

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Supplementary Material

Supplementary data (Experimental procedures, 1D and 2D NMR, HPLCESIMS spectra for **1** and **2**) associated with this article can be found, in the online version, at htt

Hig

- *Isolation of viriditin and its derivative viriditin B from Byssochlamys spectabilis*
- Variable temperature NMR indicates *cis-trans*-amid isomerism of viriditin B
- Elucidation of absolute configuration by Mosher's and Marfey's methods
- Feeding of 13C-labelled precursors reveals the biogenesis