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Induced secondary metabolites from the endophytic fungus *Aspergillus versicolor* through bacterial co-culture and OSMAC approaches

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Abstract

Two new cryptic 3,4-dihydronaphthalen-(2*H*)-1-one (1-tetralone) derivatives, aspvanicin A (**1**) and its epimer aspvanicin B (**2**), as well as several known cryptic metabolites (**3**-**8**), were obtained from the ethyl acetate extract of the co-culture of the endophytic fungus *Aspergillus versicolor* KU258497 with the bacterium *Bacillus subtilis* 168 trpC2 on solid rice medium. When *A. versicolor* was cultured axenically in liquid Wickerham medium supplemented with 3.5% DMSO, an additional three known secondary metabolites (**9**-**11**) were isolated that were lacking when the fungus was fermented on rice medium. The structures of the new compounds were elucidated using one- and two-dimensional NMR spectroscopy as well as HRESIMS. The relative and absolute configurations of **1** and **2** were determined by the combination of NMR and electronic circular dichroism (ECD) analysis aided by DFT conformational analysis and TDDFT-ECD calculations. The ECD calculations revealed that although the sign of the blue-shifted overlapping n-π* ECD transition follows the helicity rule of cyclic aryl ketones, the calculation of low-energy conformers and ECD spectra was necessary to determine the stereochemistry. All metabolites were assessed for their antibacterial and cytotoxic activities; one of the new diastereomers, compound **2**, showed moderate cytotoxic activity against the mouse lymphoma cell line L5178Y.

Keywords: *Aspergillus versicolor*; Tetralone derivatives; Absolute configuration; ECD calculations

Introduction

Endophytic fungi represent a promising and still largely untapped reservoir of biologically active secondary metabolites with potential for exploitation in the pharmaceutical industry.[1,2] *Aspergillus* is one of the oldest fungal genera known to science, having been described for the first time in 1729.[3] *Aspergillus versicolor* is widespread in different habitats (plants, marine, air dust and soil), and has the ability to survive under extreme natural environmental conditions that are usually unfavourable for growth.[4-6]

Co-cultivation of fungi with bacteria (mixed fermentation) has in many cases been shown to trigger the accumulation of new cryptic secondary metabolites or to enhance the production of constitutively present compounds through the activation of silent biosynthetic pathways.[7,8] This technique attempts to mimic natural microbial communities where fungi and bacteria co-exist and interact in a competitive manner for nutrients.[9,10] The co-cultivation of several species of *Aspergillus* with different bacteria has proved to be a powerful tool to enrich the chemical diversity of these fungi. Representative examples include: two antibacterial compounds, fumicycline A and fumicylcline B, from the co-cultivation of *A. fumigatus* with *Streptomyces rapamycinicus*,[11] and co-cultivation of the endophytic fungus *A. austroafricanus* with *Bacillus subtilis* or with *Streptomyces lividans* which led to the accumulation of the new diphenyl ether austramide.[12] In a recent study, Proksch and co-workers reported the isolation of a new cytotoxic dihydroquinolone derivative aflaquinolone H along with two new dimeric isocoumarins (6,6',9'-trinor-bipenicillisorin and 6,6'-dinor-bipenicillisorin), from the same endophytic fungus *A. versicolor* KU258497.[13] This encouraged us to conduct a co-cultivation experiment of this fungus with the bacterium *B. subtilis* with the aim to stimulate silent biogenetic gene clusters.

In addition to co-cultivation, the OSMAC (One Strain MAny Compounds) approach is considered an important technique to enhance the chemical diversity of fungal metabolites.[14] Even slight alterations of culture conditions (e.g. different temperature, culture media, light conditions, pH, carbon source, nitrogen source, culture vessels) may have a pronounced influence on the accumulation of fungal natural products.[8] Examples illustrating the usefulness of this technique include the replacement of water in solid rice media by either fruit or vegetable juices which led to an 80-fold increase in the accumulation of the new cytotoxic compound fusarielin J, as well as the biosynthesis of two new compounds, fusarielin K and L, by the endophytic fungus *Fusarium tricinctum*.[15] When the marine derived fungus *Aspergillus terreus* was grown on different media, malt agar and barley-spelt solid media were shown to induce the new cytotoxic compound 7-desmethylcitreoviridin.[16] In the present study, an OSMAC approach was applied for the cultivation of the fungus *A. versicolor* KU258497 in liquid Wickerham medium supplemented with 3.5% DMSO as a stress factor.[8]

Using co-cultivation or the OSMAC approach as tools, we succeeded in the isolation of two new dihydronaphthalenone derivatives, aspvanicin A (**1**) and its diastereomer aspvanicin B (**2**),

as well as six known cryptic metabolites including methyl orsellinate (**3**),[17] orsellinic acid (**4**),[18] cordyol C (**5**),[19] sydowiol B (**6**),[20] *p*-hydroxybenzaldehyde (**7**)[21] and fusarubin (**8**).[22] Moreover, cultivation of the fungus in liquid Wickerham medium supplemented with 3.5% DMSO yielded the known compounds sterigmatocystin (**9**),[23] infectopyrone (**10**)[24] and versiol (**11**).[25] All isolated compounds (Fig. 1) were investigated for their antibacterial, antitubercular and cytotoxic activity against the mouse lymphoma cell line L5178Y.

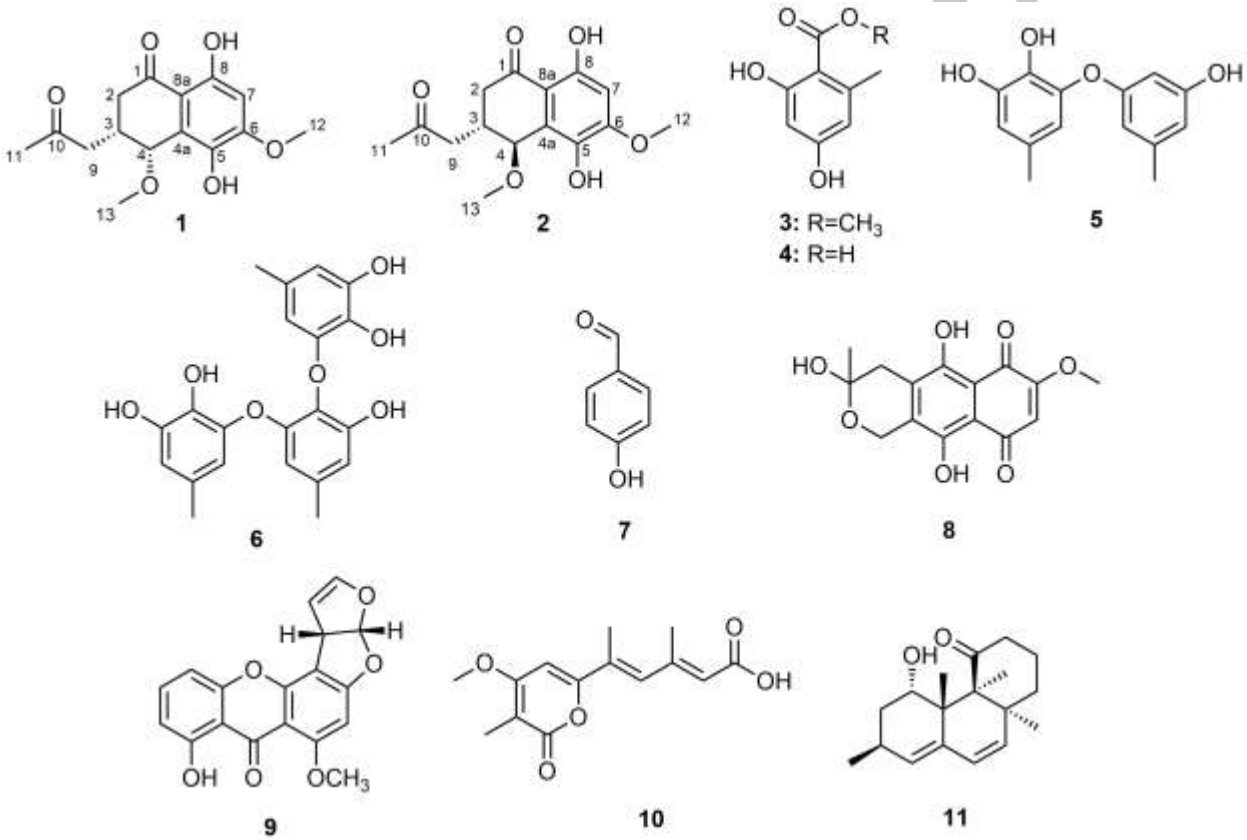

Figure 1. Structures of the isolated compounds

Results and Discussion

Compound **1** was isolated as a pale brown amorphous solid from the EtOAc extract of the fungal bacterial co-cultivation. ESI-HRMS showed a pseudomolecular ion peak at *m/z* 295.1173 $[M+H]^+$ (calculated for 295.1176 $C_{15}H_{19}O_6$) indicating seven degrees of unsaturation.

The UV spectrum showed absorption maxima at 208, 243 and 285 nm which are characteristic of dihydronaphthalenones.[26] The ^{13}C NMR data (Table 1) showed 15 different carbons grouped into seven quaternary carbons, including two carbonyl carbons (δ_C 203.2, 208.3), three oxygenated aromatic carbons (δ_C 137.6, 156.8, 158.0) and two non-oxygenated aromatic carbons (δ_C 128.0, 108.0). Moreover, the ^{13}C NMR data revealed the presence of two methoxy groups, one aromatic (δ_C 56.6) and one aliphatic (δ_C 57.1), one methyl group (δ_C 31.1), one aromatic tertiary carbon (δ_C 100.4), two aliphatic tertiary carbons, including one oxygenated (δ_C 70.7) and one non-oxygenated carbon (δ_C 35.4), and two secondary aliphatic carbons (δ_C 38.0, 45.5).

The ^{1}H-NMR data (Table 1) displayed one aromatic proton at δ_H 6.54 (s, H-7), one methyl group at δ_H 2.14 (s, H_3-11), two methoxy groups, including a methoxy substituent at an aromatic ring at δ_H 3.90 (s, H_3-12) and an aliphatic methoxy at δ_H 3.20 (s, H_3-13). Furthermore, the ^{1}H-NMR data exhibited two methylene groups [δ_H 2.34 (dd, J = 4.1, 17.3 Hz, Ha-2), δ_H 2.58 (m, Hb-2) and δ_H 2.60 (dd, J = 6.5, 17.5 Hz, Ha-9), δ_H 2.84 (dd, J = 6.7, 17.5 Hz, Hb-9)]. In addition, two methine signals were detected at δ_H 4.70 (d, J = 1.8 Hz, H-4) and at δ_H 2.49 (m, H-3). Moreover, the ^{1}H-NMR spectrum showed two hydroxyl groups, one at 8.66 (s, OH-5) ppm and a chelated OH group at 12.63 (s, OH-8) ppm.

Based on UV, ^{1}H and ^{13}C NMR data (Table 1) combined with the molecular formula obtained from ESI-HRMS, compound **1** revealed a close structural similarity to the previously reported dihydronaphthalenone derivative, 4-hydroxydihydronorjavanicin.[26] The main difference between **1** and 4-hydroxydihydronorjavanicin lies in the presence of an extra aliphatic methoxy group resonating at δ_H 3.20 (s, H_3-13). This is also reflected in the difference in the molecular weight between **1** and 4-hydroxydihydronorjavanicin amounting to 14 amu. The planar structure of **1** was confirmed *via* 2D NMR spectral analyses including ^{1}H-^{1}H COSY, HMBC and ROESY experiments (Fig. 2). The ^{1}H-^{1}H COSY spectrum displayed a solitary spin system CH_2(2)CH(3)CH_2(9)CH(4) as depicted in Figure 2. Furthermore, the HMBC spectrum (Fig. 2) revealed correlations from the aromatic proton H-7 to C-5 (δ_C 137.6), C-6 (δ_C 156.8), C-8 (δ_C 158.0) and C-8a (δ_C 108.0). The methoxy groups OCH_3-12 and OCH_3-13 were present at C-6 and C-4, respectively, as revealed by their HMBC correlations from OCH_3-12 to C-6 (along with its ROESY correlation with H-7) and from OCH_3-13 to C-4 (δ_C 70.7). Further inspection of the HMBC spectrum revealed correlations from CH_2-9 to C-4 and

C-10 (δ_C 208.3) in addition to correlations from Ha-2 to C-1 (δ_C 203.2) and from H-4 to C-2 (δ_C 38.0), C-8a and C-13 (δ_C 57.1). Moreover, Hb-2 showed a cross peak with C-4 and H-3 correlated with C-10. The position of the methyl group CH_3-11 was unambiguously confirmed by correlation of its protons with C-10 and similarly the position of the chelated hydroxyl group OH-8 was secured by key correlations with C-7 (δ_C 100.4), C-8 and C-8a, along with its ROESY correlation with H-7. The hydroxyl group OH-5 showed correlations with C-4a (δ_C 128.0), C-5 and C-6 confirming the planar structure of **1** as depicted in Figure 1. The relative configuration of **1** was deduced on the basis of coupling constants and ROESY data. Inspection of the ROESY spectrum revealed correlations between H_2-9 and both H-4 and OCH_3-13, which along with the small coupling constant (1.8 Hz) between H-4 and H-3 suggested their *cis* relationship (Fig. 2). Based on the aforementioned data, compound **1** was assigned as a new dihydronaphthalenone derivative to which the trivial name aspvanicin A is given.

Compound **2** was obtained as a pale brown amorphous solid. ESI-HRMS indicated a pseudomolecular ion peak at *m/z* 295.1178 $[M+H]^+$ (calculated for 295.1176 $C_{15}H_{19}O_6$), identical to that of compound **1**. The UV (MeOH) spectrum of **2** revealed absorption maxima (λ_{max}) at 208, 243 and 285 nm which were also identical to those of **1**, indicating that compound **2** belongs to the dihydronaphthalenone class of compounds.[26] In fact, the ^1H and ^{13}C NMR data of compounds **1** and **2** were very similar, except for the ^1H NMR chemical shifts of H_2-2, H-3 and H_2-9 (Table 1). ^1H-^1H COSY and HMBC spectral analysis (Fig. 2) confirmed the substitution pattern as shown in Figure 1.

ROESY correlations and $^3J_{H,H}$ coupling constants aided by DFT conformational analysis were used to determine the relative configuration and preferred conformation of compounds **1** and **2**. In both compounds, ROESY cross peaks from H-4 to both H-3 and H_2-9 were observed, while no ROE correlation was detected between H-4 and H_2-2. However, whereas in compound **1**, a weak ROESY correlation from OCH_3-13 to Hb-9 was found, this was not detected in compound **2**. These ROE data along with the $^3J_{H\text{-}3,H\text{-}4}$ coupling constants (1.8 Hz in **1** and 3.1 Hz in **2**) suggested that compound **1** is the *cis* diastereomer with equatorial C-3 and axial C-4 substituents, while compound **2** is the *trans* diastereomer with diaxial orientation of the C-3 and C-4 substituents, which was also confirmed by the DFT conformational analysis (*vide infra*). Compound **2** is further example of natural products, in which the small $^3J_{H,H}$

coupling constant for the methine protons of adjacent chirality centers of a condensed six-membered ring does not derive from *cis* relative configuration but instead the six-membered ring preferably adopts a conformation with *trans-diaxial* orientation of the adjacent substituents.[27] Thus, compound **2** was shown to be a diastereomer of the co-isolated aspvanicin A (**1**) to which the trivial name aspvanicin B is given.

Both aspvanicin A (**1**) and B (**2**) contain a substituted 1-tetralone (3,4-dihydronaphthalene-1(2*H*)-one) chromophore with two chiral centers at the C-3 and C-4 positions of the fused cyclohexanone ring, which offers the possibility to determine the absolute configuration and preferred conformation of **1** and **2** by ECD measurements and TDDFT-ECD calculations. This chromophore belongs to the group of cyclic aryl ketones, which also includes 2,3-dihydroquinoline-4(1*H*)-one,[27] flavanones,[28] 2-alkylchromanones,[29] and isoflavanones.[30,31] The sign of the long-wavelength n-π* cotton effect (CE) of these chromophores were correlated with the helicity of the condensed six-membered ring, according to which *M* helicity of the ring results in a negative n-π* CE. Since the preferred *M/P* helicity of the ring is governed by the chiral centers, the sign of the n-π* CE can allow the absolute configuration to be determined. Although the same correlation (helicity rule) is expected for the 1-tetralone chromophore,[32] our recent ECD calculations on conformationally restricted condensed,[33,34] and bridged[35] 1-tetralone natural products showed that auxochromic substituents such as hydroxyl or alkoxy groups on the condensed benzene ring can induce a red-shift of the π-π* transition, while a chelating 8-OH group can shift the n-π* CE to a lower wavelength. Thus, the π-π* transition becomes the highest-wavelength transition and n-π* CE overlaps with other π-π* transitions rendering determination of the absolute configuration ambiguous by the helicity rule. TDDFT-ECD calculations of 1-tetralone derivatives are recommended for 8-hydroxy derivatives and with other auxochromic substituents on the benzene ring or with conformationally flexible cyclohexenone rings.[36]

The absolute configuration of **1** and **2** was determined by thorough conformational analysis and the solution TDDFT-ECD method.[37,38] Near mirror image ECD spectra were recorded for **1** and **2**, which suggested the diastereomers have opposite helicity for the preferred low-energy conformers. The Merck Molecular Force Field (MMFF) conformational search of the arbitrarily chosen (3*S*,4*S*)-**1** and (3*S*,4*R*)-**2** resulted in 7 and 9 conformers, respectively, in a 21 kJ/mol energy window. These conformers were reoptimized at the B3LYP/6-31G(d) and

CAM-B3LYP/TZVP PCM/MeCN levels yielding 4-6 low-energy conformers over 1% Boltzmann populations (ESI). The low-energy conformers differed virtually only in the conformation of the flexible C-3 substituent and in all preferred geometries of **1** and **2**, the 4-OCH_3 group had an *axial* orientation,[27,39] while the C-3 substituent was *equatorial* in **1** and *axial* in **2** which is in full agreement with the NMR results. The *M* helicity envelop conformation was found to be the exclusive computed conformation for (3*S*,4*S*)-**1** with a torsional angle $\omega_{C4a\text{-}C4\text{-}C3\text{-}C2}$ = −58.9° (Fig. 2) and the *P* helicity conformation for **2** with a +57.5° angle (values for the lowest-energy CAM-B3LYP/TZVP PCM/MeCN conformers). ECD calculations for the conformers of (3*S*,4*S*)-**1** and (3*S*,4*R*)-**2** at various levels of theory (B3LYP/TZVP, BH&HLYP/TZVP, CAM-B3LYP/TZVP, PBE0/TZVP) gave mirror-image computed ECD spectra of the experimental one with substantially higher intensities (*c.a.* 8 fold) suggesting scalemic mixtures with an excess of the (3*R*,4*R*) enantiomer of **1** and the (3*R*,4*S*) enantiomer of **2**.[40] In Figure 3 and 4, the small experimental intensities (Δε) of the recorded ECD spectra were multiplied by 8 in order to be comparable with the computed values, which suggested that the samples are not enantiopure but scalemic mixtures with an excess of the identified enantiomers. It is interesting to note that for **2** the BH&HLYP functional reproduced the 265 nm shoulder while the B3LYP and PBE0 functionals performed better in the low-wavelength region and gave no shoulder at 265 nm.[41] For both compounds, the computed first and fourth high-wavelength transitions were found to be π-π* ones while the second and the third ones are of n-π* origin (Fig. 3 and 4). The second high-wavelength transition of (3*S*,4*S*)-**1** was derived from the tetralone carbonyl n-π* and its negative CE was in accordance with the *M* helicity of (3*S*,4*S*)-**1**. This characteristic n-π* transition overlaps with a π-π* and the side-chain n-π*, which does not allow safe application of the n-π* helicity rule for configurational assignment. The blue-shift of the n-π* CE is attributed to the hydrogen-bonding 8-OH group which chelates to the carbonyl oxygen and auxochromic 5-OH and 6-OCH_3 groups. Similarly, positive computed overlapping n-π* CE of the tetralone unit was identified as the second high-wavelength transition, which derives from the *P* helicity of (3*S*,4*R*)-**2**. Although the helicity rule of cyclic aryl ketones was found to be valid for the recent 1-tetralones, its application is not feasible for configurational assignment due to the ambiguous identification of the n-π* CE without calculations.

Table 1. ^{1}H and ^{13}C NMR data of compounds **1** and **2**.

Position	1		2	
	δ_H[a], mult. (*J* in Hz)	δ_C[a,b], type	δ_H[a], mult. (*J* in Hz)	δ_C[a,b], type
1		203.2, C		202.0, C
2	2.34, dd (4.1, 17.3)	38.0, CH_2	2.23 br d (17.8)	37.4, CH_2
	2.58, m		2.97, dd (5.4, 17.8)	
3	2.49, m	35.4, CH	2.86, m	31.0, CH
4	4.70, d (1.8)	70.7, CH	4.61, d (3.1)	72.4, CH
4-a		128.0, C		124.0, C
5		137.6, C		138.9, C
6		156.8, C		156.6, C
7	6.54, s	100.4, CH	6.54, s	99.7, CH
8		158.0, C		158.7, C
8-a		108.0, C		108.0, C
9	2.60, dd (6.5, 17.5)	45.5, CH_2	2.30, m	43.9, CH_2
	2.84, dd (6.7, 17.5)			
10		208.3, C		208.0, C
11	2.14, s	31.1, CH_3	2.01, s	29.7, CH_3
12	3.90, s	56.6, CH_3	3.89, s	56.0, CH_3
13	3.20, s	57.1, CH_3	3.28, s	56.0, CH_3
OH-5	8.66, s		8.63, s	
OH-8	12.63, s		12.59, s	

[a] Measured in DMSO-d_6 (^{1}H NMR 600 MHz and ^{13}C NMR 150 MHz)
[b] Data were assigned and confirmed by HMBC and HSQC spectra

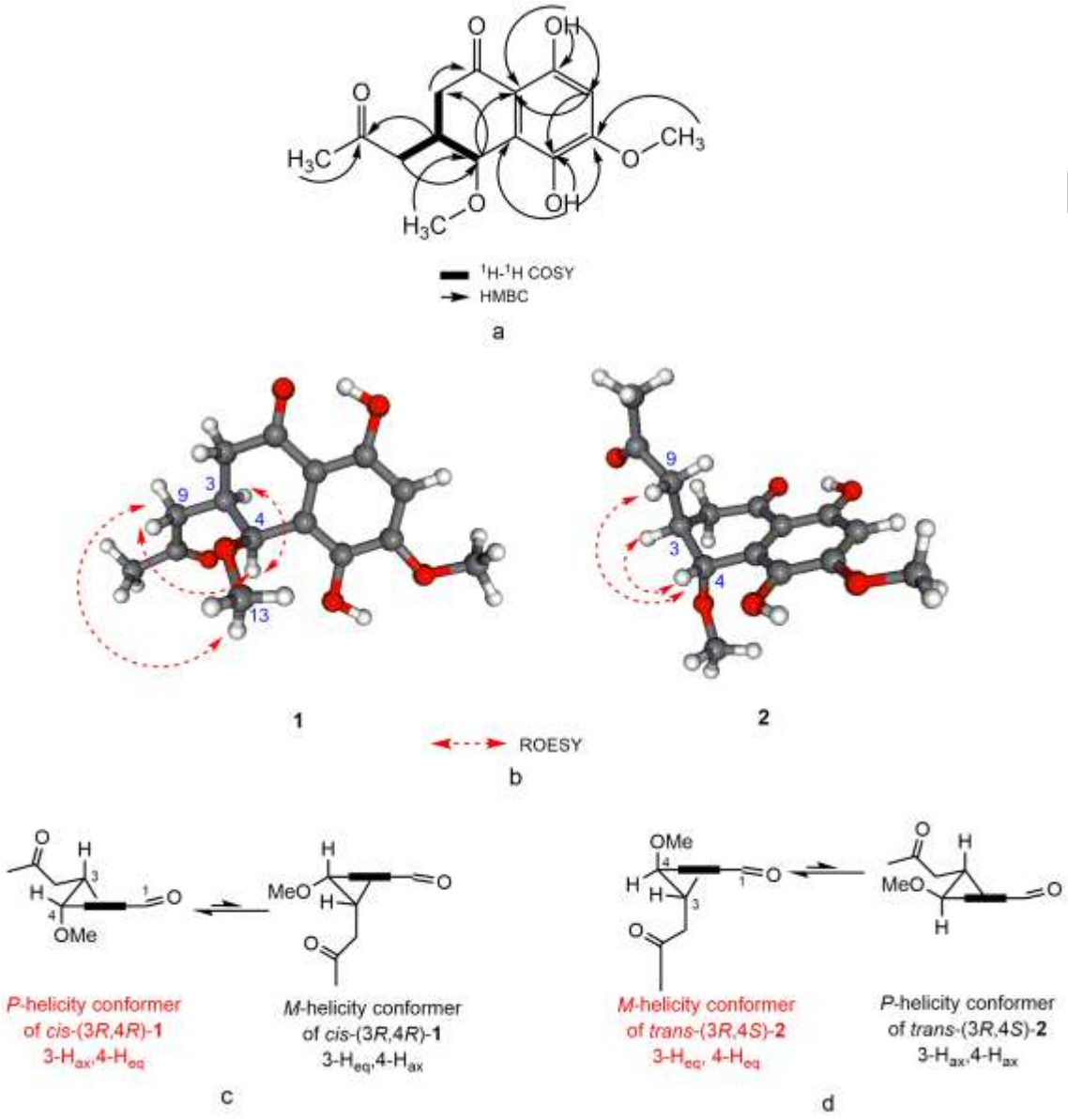

Figure 2. (**a**) Key ^{1}H-^{1}H COSY and HMBC correlations of compounds **1** and **2**. (**b**) Selected ROESY correlations of **1** and **2** (lowest-energy computed conformers). (**c**) and (**d**) Different conformers of compounds **1** and **2**.

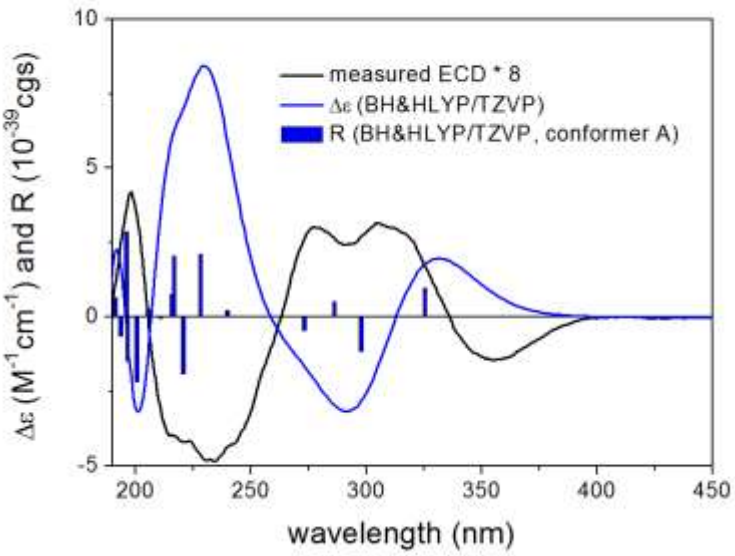

Figure 3. Experimental ECD spectrum (black) of **1** in MeCN compared with the Boltzmann-weighted BH&HLYP/TZVP PCM/MeCN ECD spectrum (blue) of (3*S*,4*S*)-**1** computed for the 5 low-energy CAM-B3LYP/TZVP PCM/MeCN conformers. The bars represent the rotational strength of the lowest-energy conformer.

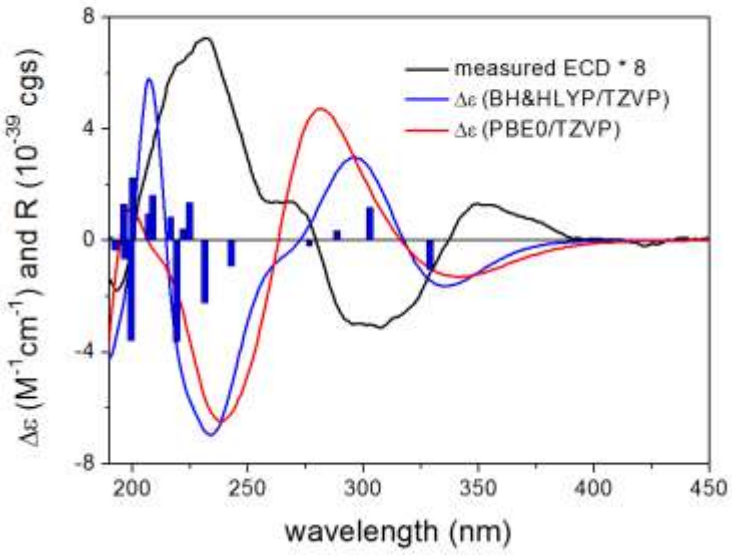

Figure 4. Experimental ECD spectrum (black) of **2** in MeCN compared with the Boltzmann-weighted BH&HLYP/TZVP PCM/MeCN (blue) and PBE0/TZVP PCM/MeCN (red) ECD spectra of (3*S*,4*R*)-**2** computed for the 6 low-energy CAM-B3LYP/TZVP PCM/MeCN conformers. The bars represent the rotational strength of the lowest-energy conformer computed at the BH&HLYP/TZVP PCM/MeCN level.

Compound **1** is closely related to the reported 4-hydroxydihydronorjavanacin, which contains a *cis* 4-OH substituent instead of the *cis*-4-OCH_3 of **1**.[26] However, the reported 4.7 Hz value for the $^3J_{3\text{-H},4\text{-H}}$ lies closer to the 3.1 Hz value of *trans*-**2** than to the 1.8 Hz of **1** and the reported NOE correlation between 2-H_{ax} and 4-H_{ax} derives from minor conformers with a diequatorial orientation of C-3 and C-4 substituents. Thus, it is likely that 4-hydroxydihydronorjavanacin has a *trans* relative configuration and similarly to **2**, the C-3 and C-4 substituents adopts mainly diaxial orientation. Our recent results emphasize that a small $^3J_{\text{H,H}}$ coupling constant of methine protons of contiguous chiral centers in a six-membered ring does not necessarily mean a *cis* relative configuration and combination with DFT conformational analysis and ECD calculation can help to deduce the stereochemistry.

The presence of compounds **1** and **2** in the crude EtOAc extract of *A. versicolor*, as detected by their retention times and UV patterns in HPLC (ESI), suggests that both compounds are true natural products and not formed from their respective demethyl derivatives during chromatographic work up.

Interestingly, the isolated metabolites (**1**-**8**) from the extract of the co-culture of *A. versicolor* with *B. subtilis* in the present study were never detected nor isolated from the axenic fungal[13] nor bacterial cultures, as it is clearly indicated in the HPLC chromatograms (ESI). These results provide a further example indicating that fungal-bacterial co-cultivation is a powerful tool which could effectively induce the production of new and cryptic secondary metabolites.

All isolated compounds were assessed for their antiproliferative activity against the mouse lymphoma cell line L5178Y using the MTT assay. Among the tested compounds, only aspvanicin B (**2**) and compound **9** showed cytotoxic activity with IC_{50} values of 22.8 and 2.2 μM, respectively, compared to kahalalide F as a standard antiproliferative agent (IC_{50} = 4.3 μM). Moreover, all compounds were evaluated for their antibacterial activities against several Gram-positive and Gram-negative bacteria, within a concentration range of 0–100 μM. Only compound **6** revealed weak antibacterial activity against *Staphylococcus aureus* (ATCC 700699) with minimal inhibitory concentration (MIC) of 50 μM (19.2 μg/mL).

In conclusion, two new dihydronaphthalenone diastereomers, aspvanicin A (**1**) and aspvanicin B (**2**), were isolated from a co-culture of the endophytic fungus *A. versicolor*

KU258497 with the bacterium *B. subtilis*, along with several cryptic secondary metabolites (**3-8**). Moreover, an OSMAC experiment afforded three further known cryptic metabolites (**9-11**).

Acknowledgments

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Supplementary data

Supplementary data (Experimental section, HPLC chromatograms, UV, MS and NMR spectral data of compounds **1** and **2**) associated with this article can be found in the online version.

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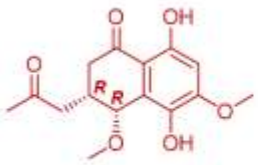

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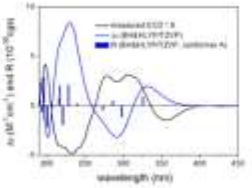
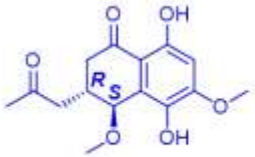


ECD Spectrum of 1



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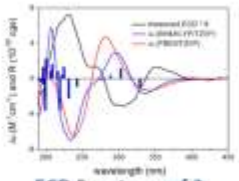
2



ECD Spectrum of 2

Highlights:

- OSMAC approach was applied for the fungus *Aspergillus versicolor*.
- Co-culture of this fungus with *Bacillus subtilis* yielded two new tetralones.
- ECD calculations were performed for the new tetralone derivatives.