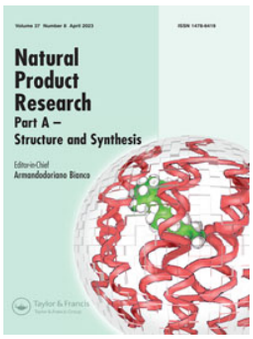

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Sulochrins and alkaloids from a fennel endophyte *Aspergillus* sp. FVL2

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ABSTRACT

The fungal endophyte *Aspergillus* sp. strain FVL2, isolated from the traditional medicinal fennel plant, *Foeniculum vulgare*, was investigated for secondary metabolites. Fermentation on rice medium followed by chromatographic separation delivered three new natural products, 7-demethyl-neosulochrin (**1**), fumigaclavine I (**3**) and *N*-benzoyl-tryptophan (**6**) together with further 14 known metabolites, 1-*O*-methyl-sulochrin-4′-sulfate, questin, laccaic acid, isorhodoptilometrin, fumigaclavine A, fumigaclavine C, fumitremorgin C, fumigaquinazoline C, tryptoquivaline J, trypacidin, 3′-*O*-demethyl-sulochrin, 1-*O*-methyl-sulochrin, protocatechuic acid, and vermelone. The chemical structures of the new metabolites were determined by NMR spectroscopy and ESI HR mass spectrometry. For fumigaclavine I, we observed the partial deuterium transfer from the solvent to the enol form with a remarkable high stereo selectivity. The discovery of the new *seco*-anthraquinone 7-demethyl-neosulochrin (**1**) revealed a second type of ring cleavage by a questin oxygenase. The discovery of diverse secondary metabolites broadens the chemical space of *Aspergillus*.

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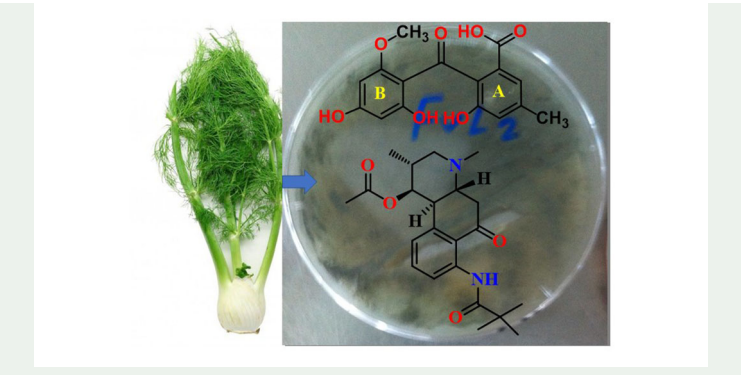
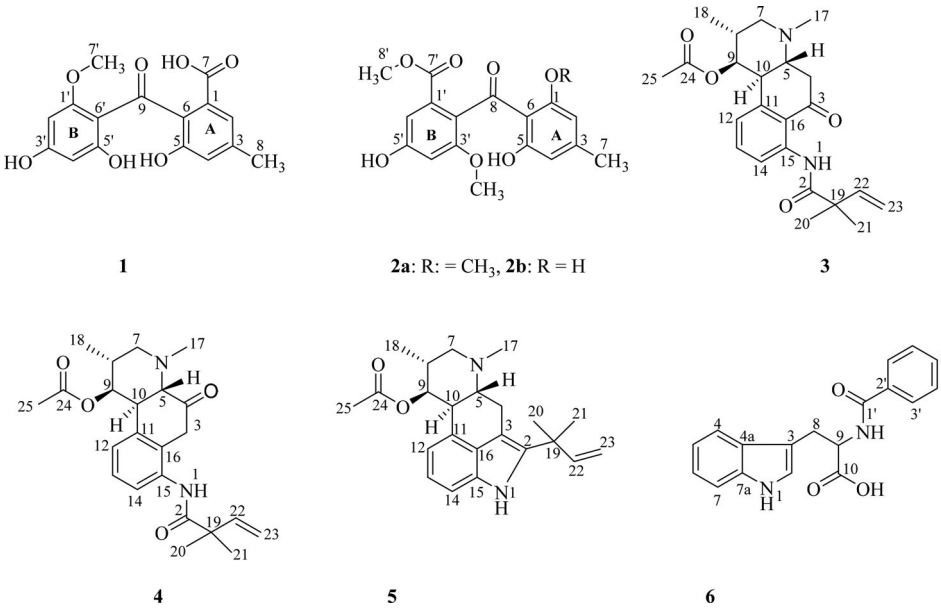


1. Introduction

Aspergillus is a fungal genus with actually more than 400 species (Cole et al. 1977), some of them having a long history of use in the food fermentation industry, e.g., *A. oryzae* (Park et al. 2017). While some species are producers of important drugs, e.g., lovastatin from *A. terreus* (McKenney 1988), others are notorious human pathogens and produce harmful mycotoxins, e.g., the fumitremorgins from *A. fumigatus* (Latgé 1999) or the aflatoxins from *A. flavus* (Cole and Cox 1981). Like many other microorganisms, some *A.* spp. are endophytes, residing in the healthy tissue of different plants. Recent evidence showed that *Aspergillus* endophytes represent an enormous source of secondary metabolites, producing butenolides, alkaloids, terpenoids, cytochalasins, phenalenones, *p*-terphenyls, xanthones, sterols, diphenyl ethers, and anthraquinone derivatives (El-Hawary et al. 2020). *Foeniculum vulgare* (Fennel) is used as a vegetable and a traditional medicinal plant with antioxidative, anti-inflammatory, estrogenic, diuretic, gynecological, antithrombotic, and antihypertensive features (Rahimi et al. 2012). Despite the previous characterization of the bioactive compounds from this plant (Mhaidat et al. 2014; Kooti et al. 2015), chemical investigation of its fungal endophytes remained unexplored.

In our search for new bioactive compounds from endophytes, the *Aspergillus* sp. strain FVL2, isolated from *F. vulgare* was analysed. LC-HRMS measurements and searching in our in-house mycotoxin library and AntiBase (Laatsch 2020) revealed the production of numerous secondary metabolites, among which several were unreported natural products. A large-scale fermentation, extraction and purification led to the discovery of three new natural products, the polyketide 7-demethyl-neosulochrin (**1**), the alkaloid fumigaclavine I (**3**), and the amino acid derivative *N*-benzoyl-tryptophan (**6**) (Figure 1), together with another 14 known metabolites (Supporting Information). These are 1-*O*-methyl-sulochrin-4′-sulfate (Han et al. 2020), questin (Chomcheon et al. 2009), laccaic acid, isorhodoptilometrin (Zhang et al. 2016; Shi et al. 2018), fumigaclavine A (Wallwey and Li 2012), fumigaclavine C (Ma et al. 2006; Abdel-Razek et al. 2018), fumitremorgin C (Lu et al. 2014; Saraiva et al. 2015), fumigaquinazoline C (Takahashi et al. 1995), tryptoquivaline J (Yamazaki et al. 1978), trypacidin (Pinheiro et al. 2013), 3′-*O*-demethyl-sulochrin (Ohashi et al. 1999), 1-*O*-methyl-sulochrin (**2a**) (Abdel-Razek et al. 2018), protocatechuic acid (Kakkar and Bais 2014), and vermelone



1 **2a**: R: = CH_3, **2b**: R = H **3**

4 **5** **6**

Figure 1. Structure of the new natural products **1**, **3** and **6** isolated in this study and the related published structures **2a**, **2 b, 4** and **5**.

(Talapatra et al. 1988; Xu et al. 2021). Subsequently, we will report on the cultivation, purification and bioactivity of **1**, **3** and **6**.

2. Results and discussion

2.1. Structure elucidation

To characterize these metabolites, the fungus was cultured on 300 g solid rice medium. The ethyl acetate extract was subjected to silica gel column chromatography, followed by separation on Sephadex LH-20 and preparative HPLC, yielding seventeen compounds including three new metabolites **1** (2 mg), **3** (1 mg) and **6** (1 mg), which were studied by NMR spectroscopy (Tables S1–S3) and HR mass spectrometry.

Compound **1** was obtained as a colorless solid with moderate polarity, showing UV absorbance and a faint orange color reaction with anisaldehyde/sulfuric acid. HRMS indicated a molecular formula of $C_{16}H_{14}O_7$. The presence of signals for four aromatic *meta*-coupled methine protons in the ^{1}H NMR spectrum indicated the presence of two aromatic systems (rings A, B, Figure 1). Moreover, signals for one ether-/ester-linked methyl (δ 3.32) and an aromatic ring-bound methyl (δ 2.32) were also observed. The ^{13}C NMR spectrum revealed 10 fully substituted sp^2 carbon atoms including one carbonyl (δ 201.7), one acid or ester carbonyl (δ 171.5), and four phenolic carbon atoms between δ 150 and 170 ppm. According to the COSY/HSQC experiments (Figure S2, Table S1), the aromatic ring attached methyl group (δ 2.32, δ 21.3) is flanked by the *meta*-coupled protons at δ 7.28 and 6.78, while the other *meta*-coupled protons (δ 5.90, 5.78) are vicinal to a phenolic carbon. The substitution pattern of ring A with a methyl group (Me-8) at C-3 (δ 139.4), and a free carboxylic acid (C-7, δ 171.5) at C-1 (δ

131.9) was confirmed by the HMBC spectrum. Me-8 exhibited correlations to C-2 and C-4, and H-2 correlated with C-7 (Figure S2). The HMBC correlations between Me-7′ and C-1′ confirmed the location of the methoxy group (δ 3.32, δ 56.0) at C-1′ (δ 165.0). The two aromatic rings were bridged by the carbonyl group C-9 (δ 201.7), which was confirmed by HMBC correlations between H-4 (ring A)/H-2′ (ring B) and C-9. Compound **1** was confirmed as a new sulochrin named 7-demethyl-neosulochrin (**1**).

A related colorless compound was identified as 1-*O*-methyl-sulochrin-4′-sulfate (**S1**), a sulochrin derivative published very recently during preparation of this manuscript (Han et al. 2020; Table S4, Figure S6).

Sulochrins are benzophenone derivatives, in which the four *ortho* positions are fully substituted. Sulochrin families are produced by different fungal genera, e.g., *Aspergillus* (Larsen et al. 2007), *Alternaria* (Cai et al. 2013) and *Penicillium* (Mahmoodian and Stickings 1964), and are displaying anti-hepatitis C virus (HCV) activity (Nakajima et al. 2013), α-glucosidase-inhibition (Dewi et al. 2018) and anti-inflammation as well as anti-asthma activity by inhibition of eosinophils (Ohashi et al. 1998).

Sulochrins **2a, 2 b** and **S1** are *seco*-anthraquinones, biosynthesized by ring cleavage of emodin with a questin oxygenase (Fujii et al. 1988). Interestingly, previous evidence showed only one type of sulochrins obtained by cleavage of the C-4a/C-10 bond in emodin, resulting in a product with an acid group formed in the dihydroxylated ring B of the product (Huang et al. 1995). Here we discovered **1** as a new isomer arising from ring cleavage between C-10/C-10a at the other side of the anthraquinone, forming the carboxylic group at ring A. The proposed biosynthesis of the different sulochrins is shown in Figure S4.

Besides the sulochrins, we isolated a new alkaloid, the fumigaclavine derivative **3**. ESI HRMS established its molecular formula as $C_{23}H_{30}N_2O_4$. According to the ^{13}C and HSQC spectra, 22 carbon signals corresponding to 23 carbon atoms were found (Table S2) and assigned to three carbonyls (δ 202.1, 177.9, 172.0), four aromatic/olefinic and four aliphatic methines, three methylene groups (δ 115.3, 57.5, 45.0), and five methyl carbons with shifts at δ 42.8, 25.2 (2 Me), 20.8, and 16.7. Remaining were three fully substituted sp^2 carbons (δ 145.1, 143.0, 120.3) and one quaternary sp^3 carbon (δ 48.0). The ^1H NMR spectrum together with COSY correlations (Figure S2) indicated three aromatic protons in a 1,2,3-position; a low-field methine proton (δ 6.11, $J = 17.4$, 10.7 Hz) and signals of an olefinic methylene group indicated a terminal ethylene fragment. The sequence from CH_2-4 to CH-5 and further to CH-10, the oxymethine CH-9, to CH-8, CH_2-7, and from CH-8 to the doublet of Me-18 was established by COSY and HMBC correlations. Three further methyl singlets were assigned in two geminal methyls at C-19, an *N*-methyl (δ 2.31) and an acetoxy group (δ 1.86). The HMBC data (Figure S2) established a 2,2-dimethylbut-3-enamide side chain, where correlations between the *gem*-dimethyl protons Me-20/Me-21 (δ 1.39) with C-2 (δ 177.9) and C-22 (δ 143.6) were seen. This C-5 sub-unit was attached to the 1,2,3-trisubstituted aromatic ring through an amide nitrogen (Figure S2). HMBC key correlations from CH_2-4 (δ 2.66, 3.13) to C-3, C-5, C-10 and C-16, from the remaining methyl groups and methine protons established the structure as the new tricyclic alkaloid **3**, a regioisomer of the structure **4** recently published for fumigaclavine I (Figure 1) (Shen et al. 2015). Our compound was further confirmed by a structure determination with COCON (Lindel et al. 1999)

and by correlation with DFT-calculated ^{13}C NHR data using SPARTAN'20 (see Supporting Information; SPARTAN'20.20 2020, Figure S7).

The relative stereochemistry of **3** was deduced by NOESY experiment (Figure S2), where correlations were observed for CH_3-18 (δ 1.30) and H-9 (δ 5.57) and between H-5 and Me-25. This confirmed the acetyl residue at C-9 and H-5 in β, and Me-18/H-10 in α-positions. The bridgehead protons H-5/H-10 are in a *trans*-orientation, thus resulting in the same relative configuration as published for fumigaclavine C (**5**).

Comparison of the experimental ECD data with the DFT-calculated theoretical spectrum indicated that formula **3** is reflecting also the absolute (5*R*,8*R*,9*S*,10*R*) configuration (Figure S3). Surprisingly, the ECD spectrum of **4** looks similar as that of the **3**-enantiomer. It should be annotated that the C-8-epimers of **3** can be distinguished sufficiently by DFT calculation of the ^{13}C NMR data (Table S5).

Fumigaclavines are derived from tryptophan through steps of prenylation, oxygenation, dihydroxylation (Han et al. 2020) and indole ring cleavage (Mutti 2012). The 2,3-double bond of indoles undergoes easily an oxidative cleavage, whereby with peracids or dioxygenases e.g., tryptophan is transformed into formylkynurenine and follow-up products (Hirata et al. 1974). Here we hypothesize a potential biosynthetic pathway for fumigaclavines which can be seen in Figure S5. After a C-prenylation on tryptophan, an oxidation occurred at the methyl group to form an aldehyde. An oxidative cyclization led to the bonds formation and decarboxylation. Further oxidation and *N*-methylation led to the formation of fumigaclavine B, which is the intermediate for fumigaclavines A, C (5) and I (3). Fumigaclavine A can be formed by acetylation of fumigaclavine B. The structural relation with the fumigaclavines in mind, **3** seems to be formed in such an oxidative cleavage way, so that it is formally a 2,3-*seco*-derivative of fumigaclavine C (**5**). For **4**, however, we could not see a similar plausible biosynthetic pathway. The NMR shifts previously published for fumigaclavine I (**4**) are agreeing with our data within the experimental error range, but do not fit on the ^{13}C NMR shifts calculated for this structure (Table S5). According tothe original report (Shen et al. 2015), the NMR data revealed a mistake in the positioning of the carbonyl group, since a clear doublet of doublet signal was observed for the carbonyl-adjacent methylene (δ 3.19, dd, 16.8, 4.2; δ 2.55, 16.8, 13.2). This confirms the direct connectivity between the methylene and H-5. We conclude therefore that both compounds are identical, and the previous structure **4** for fumigaclavine I needs to be revised to **3**.

Benzoyltryptophan (**6**) was isolated as a colorless solid. HRMS data established its molecular formula as $C_{18}H_{16}N_2O_3$. The ^1H NMR spectrum exhibited signals of nine aromatic protons, one methylene and one methine group. The 2 D NMR correlations established a 1,2-disubstituted aromatic ring and one mono-substituted benzoyl fragment (Figure S2). Besides the signals from the aromatic rings, the ^{13}C NMR spectrum revealed the presence of two carbonyl groups (δ 176.8, 167.8). Finally, HMBC correlations established structure **6** as *N*-benzoyl tryptophan (Figure S2, Table S3), which was obtained previously by synthesis **(**Zhang et al. 2018**)**. Here, we are reporting *N*-benzoyl tryptophan for the first time as a natural product.

Several other *N*-acyltryptophan derivatives have been found in nature, such as *N*-acetyltryptophan (Gebhardt et al. 2002), *N*-phenylacetyltryptophan, 4-aminocinnamoyl-tryptophan and streptomycindole (Huang et al. 2011), along with a series of cyclic

dipeptides containing tryptophan (Larsen et al. 2007). Some tryptophan derivatives were described as xanthine oxidase inhibitors (Gebhardt et al. 2002; Laatsch 2020).

2.2. Biological activity

Except for the moderate activity of trypacidin against *S. aureus* (inhibition zone 10 mm), 7-demethyl-neosulochrin, 1-*O*-methyl-sulochrin, 1-*O*-methyl-sulochrin-4′-sulfate, isorhodoptilometrin, fumigaclavine A, fumigaclavine C, fumigaquinazoline C, tryptoquivaline J, 3′-*O*-demethyl-sulochrin and vermelone were inactive in the agar diffusion assay against *S. aureus, B. subtilis, P. aeruginosa*, and *C. albicans*. Demethyl-neosulochrin (**1**), 1-*O*-methyl-sulochrin-4′-sulfate (**S1**), 1-*O*-methyl-sulochrin (**2a**), fumigaclavine I (**3**) and 3-*O*-demethyl-sulochrin were tested additionally against the human leukemia HL-60 cell line but displayed no cytotoxicity (IC_{50} >10 μM).

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded on a Bruker AMX 300, a 800 MHz Bruker Avance III HD or Varian Inova 500 spectrometer. The 800 MHz Avance III HD spectrometer was equipped with a 5 mm TCI CryoProbe (Bruker Biospin) using standard pulse sequences. ^{1}H and ^{13}C chemical shifts are referenced to solvent signals (CD_3OD: $\delta_H = 3.31$ and $\delta_C = 49.0$ ppm; DMSO-d_6: $\delta_H = 2.49$ and $\delta_C = 39.5$ ppm). Optical rotation: Polarimeter (Perkin-Elmer, model 343). ECD spectra: ECD spectra were recorded on a JASCO J-810 spectrometer equipped with a JASCO ETC-505S/PTC-423S temperature controller. UHPLC-HRMS was performed on an Agilent Infinity 1290 UHPLC system equipped with a diode array detector. UV-vis spectra were recorded from 190 to 640 nm. IR data were acquired on Bruker Alpha FTIR spectrometer using OPUS version 7.2. TLC analysis was performed on silica gel plates (Sil G/UV_{254}, 0.20 mm, Macherey-Nagel). A Biotage Isolera One Flash Chromatography system was used for flash chromatography and was performed on silica gel 60 (Merck, 0.04–0.063 mm, 230–400 mesh ASTM). Sephadex LH-20 (Amersham Biosciences, Ltd.) was purchased from Sigma-Aldrich Chemie, Steinheim, Germany. All solvents and chemicals used for HRMS and chromatography were VWR Chemicals LC-MS grade, while for extractions, the solvents were of HPLC grade (VWR Chemicals).

3.2. Isolation and characterization of the fungal isolate

The strain FVL2 was obtained from the traditional medicinal plant *F. vulgare* in Egypt. The strain is deposited at Microbial Chemistry Department, National Research Centre (NRC), Egypt. 18S rRNA gene (accession no. MW281863) was obtained and sequenced, which showed 99% similarity to *Aspergillus tubingensis* ZfF7.2. A phylogenetic tree was constructed (Figure S100). Further details regarding the fungus isolation/characterization, DNA isolation and 18S rDNA, are shown in supplementary file.

3.3. Fermentation, extraction and isolation

A spore suspension of the fungal strain was inoculated into 100 mL of ISP2 medium (malt extract 10 g/L; yeast extract 4 g/L and glucose 4 g/L) at 30 °C for 3 days as the seed culture. Of the seed culture, 5 mL were used to inoculate 1 L Erlenmeyer flasks (6 flasks) containing rice medium (50 g commercial rice, 50 mL water containing 5% peptone). The cultures were incubated for 14 days at 37 °C. Methanol was used for extraction followed by filtration under vacuum. After complete evaporation of methanol, the water phase was re-extracted by ethyl acetate. The obtained ethyl acetate extracts were concentrated *in vacuo* to dryness. The crude extract (1.63 g) was applied to fractionation using Isolera One (Biotag SNAP Cartridge, 50 g) with DCM-MeOH gradient starting from 100% DCM, with a slow increase of MeOH from 1% to 15%, and finally washed with 100% MeOH (200 mL). According to TLC monitoring, nine fractions were obtained: FI-IX. Fraction FIII was firstly separated by Sephadex LH-20 (CH_2Cl_2/40%CH_3OH) followed by PTLC (CH_2Cl_2/3% CH_3OH) and then purified again by Sephadex LH-20 (CH_2Cl_2/40%CH_3OH), affording trypacidin (**S9**, 18.4 mg), 3′-*O*-demethyl-sulochrin (**S10**, 2.5 mg), 1-*O*-methyl-sulochrin (**2a**, 34.6 mg) and fumigaquinazoline C (**S7**, 2.5 mg). Fraction IV afforded questin (**S2**, 13.6 mg) in addition to vermelone (**S12**, 2 mg). Fractions FV and FVI were combined and subjected to Sephadex LH-20 (CH_2Cl_2/50% CH_3OH) purification affording isorhodoptilometrin (**S4**, 1.4 mg). A subfraction residue of the combined fractions FV-FVI was further purified by HPLC using CH_3CN-H_2O system resulting in fumitremorgin C (**S6**, 1 mg), 1-*O*-methyl-sulochrin-4′-sulfate (**S1**, 2 mg) and *N*-benzoyl-tryptophan (**6**, 1 mg). Purification of fraction VIII on Sephadex LH-20 (CH_3OH) afforded tryptoquivaline J (**S8**, 15 mg). FVIII was applied to HPLC (CH_3CN-H_2O) affording fumigaclavine C (**5**, 5 mg), 7-demethyl-neosulochrin (**1**, 2 mg) and fumigaclavine I (**3**, 1 mg), protocatechuic acid (**S11**, 2 mg). Fraction IX was purified by Sephadex LH-20 (CH_3OH) leading to laccaic acid (**S3**, 6.6 mg) and fumigaclavine A (**S5**, 3.2 mg).

3.3.1. 7-Demethyl-neosulochrin (1)

Colorless solid; UV (CH_3CN/H_2O) λ_{max} 284, 208 nm; IR (ATR) ν_{max} 3547, 3417, 2899, 1716, 1469, 1272, 1205, 1168, 1113 cm^{-1}; ^1H NMR (CD_3OD) (Table S1); ^{13}C NMR (Table S1); (+)-ESI HRMS *m/z* 319.0819 $[M+H]^+$ (calcd for $C_{16}H_{15}O_7$, 319.0812, Δ 2.1 ppm).

3.3.2. Fumigaclavine I (3)

Colorless solid; UV (CH_3CN/H_2O) λ_{max} 348, 270, 212 nm; IR (ATR) ν_{max} 3205, 2931, 1730, 1694, 1650, 1579, 1468, 1405, 1238 cm^{-1}; $[\alpha]_D^{20}$ −29.66 (c 0.21, CH_3OH); ^1H NMR (CD_3OD) (Table S2); ^{13}C NMR (Table S2); (+)-ESI HRMS *m/z* 797.4498 $[2M+H]^+$ (calcd for $C_{46}H_{61}N_4O_8$, 797.4484, Δ 1.8 ppm).

3.3.3. N-Benzoyl-tryptophan (6)

Colorless solid; UV (CH_3CN/H_2O) λ_{max} 218 nm; IR (ATR) ν_{max} 850 cm^{-1}; ^1H NMR (CD_3OD) (Table S3; ^{13}C NMR (Table S3); (+)-ESI HRMS *m/z* 617.2389 ($[2M+H]^-$ (calcd for $C_{36}H_{31}N_4O_6$, 617.2395, Δ 0.9 ppm).

3.4. Biological activity testing

3.4.1. Antimicrobial assay

The antimicrobial activity was determined by disk diffusion bioassays (Bauer et al. 1966) against a set of test organisms including Gram-positive bacteria (*S. aureus* 8325, *B. subtilis* ATCC6051), Gram-negative bacterium (*P. aeruginosa* Pao1) and a fungus (*C. albicans* IBT654). Media for bacteria: 3 g meat extract, 5 g Bacto Pepton, 5 g Glucose, 1 L tap water, pH 7.3-7.5, 18 g agar; medium for *C. albicans* IBT 654: 40 g glucose, 10 g Bacto Pepton, 1 L tap water, pH 4.8, 36 g agar.

3.4.2. Cytotoxicity assay

Cytotoxicity against the human cell line HL-60 was evaluated using Alamar Blue (Thermos Scientific, Kansas, USA). The assay (Hamid et al. 2004) was performed in 96 well plates (Costar 3595, Corning, New York, USA), with an assay volume of 200 µL. The software Prism 5.03 was used for data analysis (GraphPad Software, USA).

3.5. Ab initio calculations

DFT calculations of NMR, OR, and ECD data were performed as described previously, however, SPARTAN'20 was used now, applying the automatic NMR routine (Shaaban et al. 2019).

4. Conclusions

Three new natural products, 7-demethyl-neosulochrin (**1**), fumigaclavine I (**3**) and *N*-benzoyl-tryptophan (**6**) were isolated from a fennel fungal endophyte. The absolute configuration of fumigaclavine I (**3**) was determined by CD spectra and DFT calculations. The discovery of the new *seco*-anthraquinone 7-demethyl-neosulochrin (**1**) revealed a second type of ring cleavage by a questin oxygenase. Our work has broadened the chemistry of *Aspergillus* as an important source of metabolites.

Disclosure statement

No potential conflict of interest was reported by the authors.

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