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NOTE

1H,1′H-[3,3′]biindolyl from the terrestrial fungus *Gliocladium catenulatum*

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Gliocladium is a genus known for its production of bioactive secondary metabolites. Terpenes,[1,2] sulfur-containing diketopiperazines[3] and polyketides[4] are just a few examples of the diverse structural and biological activity of these compounds. Some *Gliocladium* strains are also employed commercially as biocontrol agents in the field against pests.[5]

American foulbrood is a disease of the larvae and pupae of honeybees caused by the bacterium *Paenibacillus larvae*, which produces important economical losses. Owing to the lack of effective treatments, apiculture producers employ commercial antibiotics generating known consequences such as acquisition of resistant mechanisms to those drugs and introduction of contamination to the beehives and honey and other products of human consumption. For the above reasons, we began the search for specific antibiotics against *P. larvae* produced by fungal cultures in order to improve this productive activity.

In this context, while screening fungal cultures of diverse origin for new bioactive metabolites,[6,7] we investigated a strain of *Gliocladium catenulatum*. The mycelial extract of this fungus contained a previously undescribed biindol metabolite **1**, which showed antimicrobial activity against *P. larvae*, an entomopathogenic bacterium that attacks the larvae of the honey bee *Apis melifera*. This report deals with the fermentation, isolation and structural analysis of this metabolite and two other known gliocladins.

The *G. catenulatum* Gilman and Abbot strain was isolated from vertic soil sediments from a lettuce plantation in Las Heras, Buenos Aires, Argentina. Strains were isolated by the soil particle washing method.[8] The strain was deposited in the BAFC culture collection (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) under the accession number BAFC 3584.

A well grown agar slant of *G. catenulatum* was used to inoculate five 250 ml Erlenmeyer flasks containing 75 ml of malt extract medium containing 30 g of malt extract (Oxoid, Cambridge, UK) and 5 g of bacteriological peptone (Oxoid) per liter. After 1 week, the content of one Erlenmeyer flask was transferred to inoculate 1 l of the same media in each of the five 5 l Erlenmeyer flasks. The fermentation was carried out at 25 °C for 20 days under dark static conditions.

The fermentation broths were filtered and the mycelium was extracted with ethanol and ethyl acetate. The extracts were combined, yielding a crude extract of 15 g by evaporation under reduced pressure.

The mycelium crude extract was subjected to vacuum chromatography on RP-18, using a gradient elution from 100% H_2O to 100% methanol. The fourth fraction (75:25 methanol:H_2O), which was the only active one, was purified employing HPLC (Thermo Separation Products, Waltham, MA, USA, spectra series P100; column: YMC RP-18, 5 μm, 22.5 × 2.5 cm^2; mobile phase: MeOH–H_2O (65:35), 5 ml min^{-1}; detection: UV 220 nm, refractive index) yielding pure compound **1** (5 mg) and a mixture of **2** and **3** (Figure 1). Pure compounds **2** (2 mg) and **3** (2 mg) were obtained by preparative TLC on silica gel (CH_2Cl_2:MeOH, 97:3) from that mixture.

The antibiotic activity against *P. larvae* was determined by the agar diffusion method,[9] using MPYGP-thiamine 0.01% medium. The *P. larvae* strain C, used in this study, was isolated from beehives at the province of Buenos Aires, Argentina, and classified and maintained at the Laboratorio de bacteriología, Departamento de Producciión Animal, INTA, EEA Balcarce, Argentina.[10] An inoculum density of 10^8 bacteria per ml was used. The incubations were carried out at 37 °C in 10% CO_2. Oxytetracycline was used as a positive control (inhibition halo diameter: 17 mm, 50 μg per disk; MIC 0.5 μg per disk).

The molecular formula of compound **1** was determined as $C_{16}H_{12}N_2$ according to the HR-ESI and HR-EI mass spectra (MicrOTOF-Q II, Bruker, Billerica, MA, USA), where ions $[M+H]^+$ and $M^{+\bullet}$, were observed at *m/z* 233.1072 and at *m/z* 232.1000, respectively. The ^1H NMR spectrum (Avance II, Bruker, 500.13 MHz for ^1H, $CDCl_3$–CD_3OD, 95:5) was simple with only five signals: a doublet at δ 7.85, a singlet at δ 7.51, a doublet at δ 7.47 and two double doublets at δ 7.22 and 7.14, all integrating apparently to one proton each.

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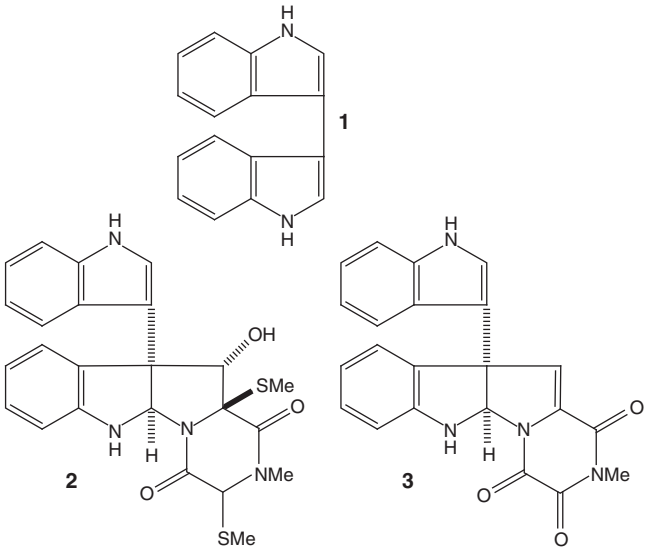

Figure 1 Isolated compounds.

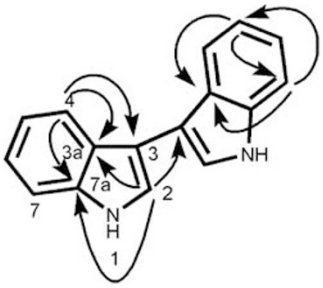

Figure 2 Heteronuclear multiple bond coherence correlations for compound 1.

The ^{13}C NMR spectrum showed five signals from aromatic methines at δ 121.9, 121.8, 120.2, 119.4 and 111.5 and three quaternary carbons at δ 136.7, 126.8 and 110.8.

A COSY experiment established the existence of a system of four consecutive methines, and the heteronuclear multiple bond correlation experiment showed correlations between the protons and the quaternary carbons, confirming an indole nucleus with a quaternary carbon at position 3, as shown in Figure 2 (for simplicity, selected correlations are shown on separate rings).

Taking the molecular formulae and the number of protons in the NMR spectra into account, a homodimeric symmetric structure was assumed. Furthermore, MS^2 experiments on $[M+H]^+$ showed a fragment *m/z* 117.058 $[C_8H_7N]^+$ to be present at a very low relative abundance (3%), suggesting that the dimer was not formed just from an association of monomers in the solution or in the ESI source, common features of ESI analysis. An atmospheric pressure photoionization mass spectrum was performed to confirm this assumption as insource dimerization is not expected, and the ions $[M+H]^+$ and $M^{+\bullet}$ were observed at *m/z* 233.1074 and at *m/z* 232.1000, respectively.

The structure of compound **1** was therefore elucidated as 3,3′-biindole.

This metabolite showed very specific activity against *P. larvae*, the bacterial pathogen of honey bee larvae, with an inhibition halo diameter of 15 mm (50 µg per disk) and MIC 5 µg per disk. This bacterium causes American fouldbrood disease, which seriously reduces honey production, and for which there are very few permitted (with restrictions) commercial antibiotics.

The spectroscopic data (2D NMR, HR-ESI-MS) of compounds **2** and **3** were identical to the known gliocladins A and C.[11,12] In fact, there are some minor discrepancies between the NMR signal assignments of natural and synthetic gliocladin C, and our data were most similar to the synthetic variant.[12]

The antibiotic activity of the crude extracts and pure compounds **1–3** were also determined against *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) by the agar diffusion method at 100 µg per disk and 50 µg per disk for extracts/fractions and compounds, respectively, and against the phytopathogens *Fusarium virguliforme*, *Colletotrichum truncatum* and *Macrophomina phaseolina* by bioautography on TLC method.[13] All the isolated compounds and the extracts were inactive in these assays.

Compound **1** has not been previously reported as a natural product, although it is known as a synthetic compound.[14] All the spectroscopic data of **1** were in full agreement with the synthetic compound. It is noteworthy that there are a few halogenated natural 3,3′-biindoles formerly reported, isolated from the cyanobacterium *Rivularia firma*[15] and beetroot, *Beta vulgaris*.[16]

On the other hand, gliocladins A and C have been isolated from a *G. roseum* found on the sea hare *Aplysia kurodai*.[11] These compounds have shown cytotoxicity against murine P388 lymphocytic leukemia cells. This cytotoxic activity is a well known property of the epipolythiodioxopiperazine (ETP) class of fungal toxins, in which the presence of a sulfur bridge as a structural feature allows the conjugation to proteins and/or the generation of reactive oxygen species via redox cycling.[17]

Compound **2** is obviously derived from an ETP compound, such as chaetomin or the verticillins, and compounds **1** and **3** are probably metabolites produced from **2**. The biosynthesis of ETPs has not been well studied and their primary physiological role is not yet known, although for several fungi, the ability to produce ETPs is associated with their causing animal and plant diseases.[17]

Production of ETPs by fungal species is not a universal phenomenon, although some generalizations may be made. For example, verticillins are produced by Sordariomycetes and Eurotiomycetes, and the closely related molecule chaetomin is produced by Sordariomycetes and Dothideomycetes.[17] *G. catenulatum* is an anamorph of *Hypocrea* Fr., classified within the Sordariomycetes group, so that chaetomin or the verticillins may be the biogenetic precursors of 3,3′-biindol. Verticillin D has previously been isolated from a *G. catenulatum* strain.[18]

Despite the expectation that marine fungal species produce different and unique metabolites compared with terrestrial ones, the isolation of compounds **2** and **3** in this study would indicate that the production of these compounds is more species specific than the source of the strain. Moreover, it has recently been observed that for gliotoxin isolated from marine strains, the surrounding high salt concentration just stimulates the release of toxin.[19]

The polyketide-derived antibiotic TMC-151, previously described as a specific metabolite of *G. catenulatum*,[20] was not isolated in this work.

In summary, two ETP-derived compounds, **2** and **3**, and 3,3′-biindol, previously undescribed as a naturally occurring product, which has specific antibacterial activity against the honey bee enthomopathogenic bacterium *P. larvae*, were all isolated and identified from a culture of the fungus *G. catenulatum*. This is the first report of the isolation of these compounds from this species.

Compound **1**: *3,3′-bisindol.* White amorphous powder; m.p. 273–274 °C. UV λ_{max} $CHCl_3$, nm (log ε): 244 (4.34). IR (KBr) ν_{max}, cm^{-1}: 2917 (NH), 1463 (CN), 741. ^{1}H NMR (500.13 MHz, $CDCl_3$–CD_3OD, 95:5): δ 7.85 (2H, d, J=7.9 Hz, H-4/4′); 7.51 (2H, s, H-2/2′); 7.47 (2H, d, J=8.1 Hz, H-7/7′); 7.22 (2H, br, t, J=8.1 Hz, H-6/6′); 7.14 (2H, dd, J=7.9 Hz, H-5/5′). ^{13}C NMR (125.13 MHz, $CDCl_3$–CD_3OD, 95:5): δ 136.7, C-7a/7′a; 126.8, C-3a/3′a; 121.9, C-6/6′; 121.8, C-2/2′; 120.2, C-4/4′; 119.4, C-5/5′; 111.5, C-7/7′; 110.8, C-3/3′. EI-HR-MS m/z 232.1000 $M^{+\bullet}$ (calcd for $C_{16}H_{12}N_2$, 232.0995, Δ −2.1 p.p.m.); ESI-HR-MS m/z 233.1072 $[M+H]^+$ (calcd for $C_{16}H_{13}N_2$, 233.1073, Δ 0.6 p.p.m.); atmospheric pressure photoionization HR-MS m/z 233.1074 $[M+H]^+$ (calcd for $C_{16}H_{13}N_2$, 233.1073, Δ −0.5 p.p.m.) and 232.1000 $M^{+\bullet}$ (calcd for $C_{16}H_{12}N_2$, 232.0995, Δ −2.1 p.p.m.). Atmospheric pressure photoionization MS/MS 233.1 u, CE 15 eV, m/z: 206.09 $[M+H\text{-}CNH]^+$,[6] 117.05 $[C_8H_7N]^+$.[2]

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