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UV-guided screening of benzodiazepine producing species in *Penicillium*

Thomas Ostenfeld Larsen[a,*], Karla Frydenvang[b], Jens Christian Frisvad[a]

[a]*Mycology Group, Department of Biotechnology, Building 221, Technical University of Denmark, DK-2800 Lyngby, Denmark*

[b]*Department of Medicinal Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, Denmark*

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Abstract

The benzodiazepine sclerotigenin (auranthine B) recently described as a metabolite of *Penicillium sclerotigenum*, has been isolated as the major metabolite from an isolate of *P. commune*. The structure of sclerotigenin was established by a single-crystal X-ray diffraction study and by NMR spectroscopy. UV-guided screening for benzodiazepine production by other penicillia revealed that sclerotigenin was also produced by isolates of *P. clavigerum*, *P. lanosum*, *P. melanoconidium*, *P. sclerotigenum* and *P. verrucosum*. Sclerotigenin was detected both intra- and extracellularly. Apparently, *P. aurantiogriseum* is the only auranthine producing species in genus *Penicillium*.

Keywords: *Penicillium*; Benzodiazepines; Sclerotigenin; Auranthine; Chemotaxonomy

1. Introduction

For more than 20 years we have studied the taxonomy and classification of species in genus *Penicillium* from a combined morphological and chemical approach (Frisvad and Filtenborg, 1983, 1989; Larsen and Frisvad, 1995). In these studies secondary

* Corresponding author. Tel.: + 45-45-25-26-32; fax: + 45-45-88-49-22.
E-mail address: tol@ibt.dtu.dk (T. Ostenfeld Larsen)

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metabolites are extracted in micro-scale (Filtenborg and Frisvad, 1980; Smedsgaard, 1997) and analysed and identified primarily by HPLC coupled to UV–VIS detection or ES-MS (Frisvad, 1987; Svendsen and Frisvad, 1994; Smedsgaard and Frisvad, 1997).

More recently, we have initiated UV-guided isolation of metabolites of physiological or chemotaxonomical significance with some focus on amino acid derived metabolites, like diketopiperazines and quinazolines (Sørensen et al., 1999; Larsen et al., 1998b, 1999). Due to the very similar UV characteristics of quinazolines and benzodiazepines our attention was drawn to a possible novel benzodiazepine metabolite ("auranthine B"), produced by an isolate of *P. commune*. The UV-spectrum of auranthine B was almost identical to the UV spectrum of auranthine from *P. aurantiogriseum* (Fig. 1, Yeulet et al., 1986).

2. Materials and methods

A total of 53 *Penicillium* isolates (Table 1), all obtained from the Culture Collection at the Department of Biotechnology (IBT), Technical University of Denmark were used in the present study.

Penicillium commune (IBT 3427) was cultured for 14 days in the dark on 200 SYES agar plates corresponding to 4 l medium (Svendsen and Frisvad, 1994). The plates were extracted repeatedly with EtOAc (2 l) to give a crude extract (2.8 g) after

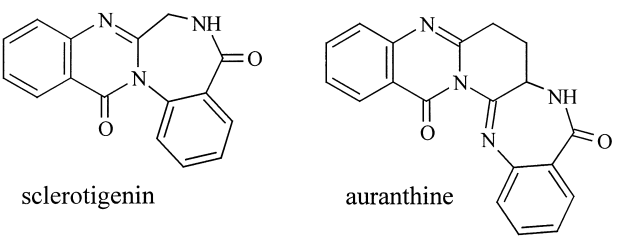

Fig. 1. Structure of sclerotigenin and auranthine.

Table 1
Penicillium isolates screened for benzodiazepine production

P. aurantiogriseum: IBT 3992, 5134, 5268, 11301, 11325, 12834, 12836, 13169.
P. commune: IBT 3427, 6200, 10727, 10837, 10924, 12807, 16710, 18102.
P. clavigerum: IBT 4899, 5523, 6336, 14991, 14993, 19355, 19361, 21512.
P. lanosum: IBT 13448, 15554, 18774, 19190, 19714, 19718, 19933, 19992.
P. melanoconidium: IBT 3928, 4107, 6672, 6794, 11406, 14367, 15448, 15983.
P. sclerotigenum: IBT 12466, 13826, 13938, 14346, 15061.
P. verrucosum: IBT 5252, 6734, 14248, 14249, 14257, 14875, 15212, 21025.

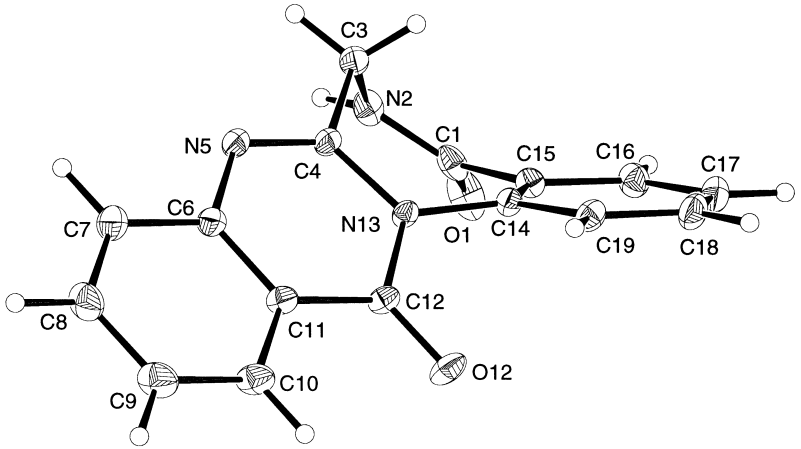

Fig. 2. Perspective drawing (Johnson, 1976) of sclerotigenin. Displacement ellipsoids enclose 50% probability. Hydrogen atoms are represented by spheres of arbitrary size. Bond lengths and angles are in agreement with expected values (Allen et al., 1995). The conformation of the molecule is dominated by the planar parts of the compound, i.e. the aromatic moieties and the amide moieties. The seven-membered ring adopts a C_S boat conformation (Spek, 1990). In the crystal packing a hydrogen bond is observed from the amide hydrogen atom (N2–H) to the amide carbonyl oxygen atom (O12) of a symmetry related molecule. The packing shows layers of hydrogen bonded molecules and the layers are held together by van der Waals interactions.

evaporation of the solvent. 620 mg of pure sclerotigenin was obtained from chromatography on a Merck Lichroprep Si (25 mm × 310 mm, 40–63 μm) column using CH_2Cl_2–MeOH (97 : 3) as mobile phase at 16 ml/min flow rate. Only part of the 620 mg of the pure sclerotigenin could be dissolved in 100 ml boiling MeOH. The MeOH phase was left in the refrigerator overnight to give more than 100 mg of colourless prisms.

A single crystal was used to collect X-ray diffraction data on an Enraf-Nonius CAD-4 diffractometer using graphite monochromated Mo K_α radiation. Data were processed using the programs of Blessing (DREADD) (Blessing, 1987, 1989, 1997) (details in deposited material).

The structure of sclerotigenin (Fig. 2) was solved by direct methods using the programme SHELXS97 (Sheldrick, 1990, 1997a) and refined using the programme SHELXL97 (Sheldrick, 1997b). Full matrix least-squares refinement on F^2 was performed, minimizing $\sum w(F_o^2 - F_c^2)^2$, with anisotropic displacement parameters for the non-hydrogen atoms. The positions of the hydrogen atoms were located from intermediate difference electron density maps and refined with fixed isotropic displacement parameters. The refinement (223 parameters, 5423 reflections) converged at $R_F = 0.042$, $wR_{F^2} = 0.111$ for 4331 reflections with $F_o > 4\sigma(F_o)$; $w = 1/[\sigma(F_o^2) + (0.0689P)^2 + 0.24P]$, where $P = (F_o^2 + 2F_c^2)/3)$; $S = 1.020$. In the final difference Fourier map maximum and minimum electron densities were 0.49 and -0.23 e $Å^{-3}$, respectively.

The ^{13}C NMR data, recorded at 100.6 MHz (DMSO-*d6*: 167.1, 161.0, 154.9, 146.2, 135.2, 133.5, 130.8, 130.7, 129.8, 128.8, 128.6, 127.6, 127.1, 127.0, 121.1 and 46.3 ppm), were in keeping with the data reported by Joshi et al. (1999).

The 53 isolates of *P. aurantiogriseum*, *P. commune*, *P. clavigerum*, *P. lanosum*, *P. melanoconidium*, *P. sclerotigenum* and *P. verrucosum* were cultivated on CYA and YES, and screened for production of benzodiazepines, both inside and outside of colonies, using the agar plug extraction procedure and HPLC analysis as described by Smedsgaard (1997). Retention indices (RI) of fungal metabolites were calculated according to Frisvad and Thrane (1987).

3. Results and discussion

HPLC analysis confirmed that auranthine (RI = 835) is consistently produced by *P. aurantiogriseum* as reported by Lund and Frisvad (1994). According to our knowledge no other penicillia is capable of producing auranthine.

Sclerotigenin (RI = 705) was found to be produced by isolates in six different *Penicillium* species. All investigated isolates of each of the three species *P. sclerotigenum*, *P. lanosum* and *P. melanoconidium* produced sclerotigenin (see Table 1).

However, sclerotigenin was inconsistently produced by *P. verrucosum*, *P. clavigerum* and *P. commune*. Only four of the eight investigated *P. verrucosum* isolates (IBT: 6734, 14248, 14257, 21025), two of the *P. clavigerum* isolates (IBT: 4899, 19355) and just one of the *P. commune* isolates (IBT 3427) were found as producers.

Besides sclerotigenin, IBT 3427 among others produced cyclopiazonic acid, cyclopaldic acid, palitantin and a series of non-characterized metabolites belonging to the same biosynthetic family ("metabolite-I analogues"), all typical metabolites for *P. commune* (Lund, 1995). Likewise, the four sclerotigenin producing *P. verrucosum* isolates had a typical profile of secondary metabolites for the species, including verrucolone (Larsen et al., 1998a), ochratoxin A (Frisvad and Filtenborg, 1989), together with quinazoline (Larsen et al., 1999) and metabolite-I analogues. In addition to that IBT 15212 produced citrinin. The two sclerotigenin producing *P. clavigerum* isolates also produced patulin, asperfuran, penitrem A, lichexanthone and TAN-1612. These latter metabolites are typical for *P. clavigerum* and were also produced by the six other *P. clavigerum* isolates investigated here.

Such an inconsistent production of a major secondary metabolite, as seen for sclerotigenin production by isolates of *P. commune*, *P. verrucosum* and *P. clavigerum* in this study, is in general atypical. Usually, when numerous *Penicillium* isolates are compared for metabolite production in an investigation (Frisvad and Filtenborg, 1989; Frisvad et al., 1998; Lund and Frisvad, 1994), most isolates are very consistent in their production of secondary metabolites. However, since all sclerotigenin producers of *P. commune*, *P. verrucosum* and *P. clavigerum* were demonstrated to produce several metabolites normally produced by the three species, and at the same time also had the expected morphological features, there is no reason to propose that they should be reclassified as new species.

The production of large quantities of a secondary metabolite in a few isolates of a species may, on the other hand, be an indication of a new speciation and so is of great interest. For example the isolate of *P. commune* producing sclerotigenin was isolated from acorns in Spain where insect interaction is possible, whereas most other *P. commue* isolates are associated to cheese (Lund et al., 1995) where mite interaction rather than insect interaction is possible.

Sclerotigenin was first isolated from sclerotia of *P. sclerotigenum* and was found to have antiinsectan activity, which was why Joshi et al. (1999) suggested sclerotigenin to play a role in the longevity of sclerotia. In this study one isolate of each of the six *Penicillium* species was investigated for appearance of sclerotigenin both intra- and extracellularly. All isolates were found to contain large amounts of sclerotigenin in their spores (or mycelium). None of the investigated *P. sclerotigenum* isolates (or isolates of the five other studied species), produced sclerotia. Sclerotigenin was also found in relatively large extracellular concentrations in *P. commune*, *P. sclerotigenum* and *P. lanosum* and in minor extracellular amounts in *P. clavigerum*, *P. melanoconidum* and *P. verrucosum*. This was surprising since pure sclerotigenin was difficult to dissolve in many organic solvents and in particular in water.

Since the biological activity of sclerotigenin has already been demonstrated our results indicate that sclerotigenin may also play a role in the chemical defense of both growing mycelium and spores.

4. Deposited material

Tables for the compound sclerotigenin list details for data collection, processing and refinement, final atomic coordinates, equivalent isotropic displacement parameters, anisotropic displacement parameters for non-hydrogen atoms, and a full list of bond lengths, bond angles, torsion angles and hydrogen bond dimensions and list of structure factors. The crystallographic CIF file has been deposited at the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (Deposition no. CCDC 134800).

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