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# Novel diterpenes with potent conidiation inducing activity

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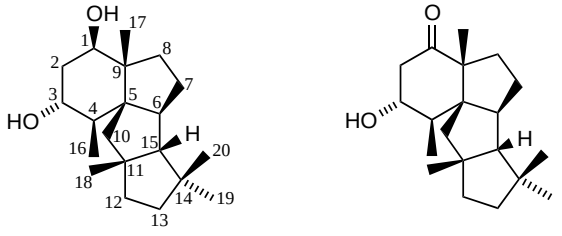
**Abstract**—The isolation and structure determination of conidiogenol and conidiogenone, tetracyclic diterpenes with a novel carbon skeleton, from extracts of the fermentation broth of *Penicillium cyclopium* is reported. Conidiogenol and conidiogenone are potent and selective inducers of conidiogenesis in *P. cyclopium* in liquid culture, and relay information about the environmental conditions to the producing organism. 

## 1. Introduction

Fungi grow in the form of filaments (hyphae) which penetrate solid substrates by force and by enzymatic hydrolysis. However, they are also able to disperse by aerial or aquatic means, as asexual spores called conidia. The process of conidiogenesis[1] is widespread, and has received considerable attention by mycologists and molecular biologists over the last few decades. The mechanisms responsible for the control of conidiogenesis have become well understood.[2] However, the triggering system of this phenomenon has remained elusive, although a number of proposals have been made.[3–7] Nevertheless, the understanding of this aspect of conidiogenesis is relevant since it could provide tools to inhibit conidiation in cases when fungal conidia spread disease in plants, animals or humans, or to induce conidiogenesis artificially in instances when this is of interest.

The fact that conidiogenesis was induced faster in spent medium, in which the fungus already had grown, compared to new medium, suggested that a compound produced during the fermentation was involved in the triggering of the process in some Penicillia.[8,9] Fractionation of extracts of the fermentation broth of *Penicillium cyclopium* (prepared according to the experimental part) and the testing of the fractions for conidiogenesis inducing capability eventually led to the isolation of two active compounds, which we have named conidiogenol **1** and conidiogenone **2** (see Fig. 1). Both compounds, which are related tetracyclic diterpenes, are potent and selective inducers of conidiogenesis in *P. cyclopium*, in liquid culture, under non-nutrient limiting conditions. Only 20 ng **1** or **2** per ml of medium is sufficient for full induction of conidiogenesis.[10] Although conidiogenol **1** is as potent as conidiogenone **2**, it is slower acting and will induce conidiogenesis only after a delay of several hours. It may be assumed that conidiogenone **2** is the true or ultimate inducer and the delay observed with conidiogenol **1** reflects the necessity to oxidise **1** to **2** in the medium. Both appear to be produced continuously in small amounts by the mycelium and are passed into the external medium. A spent culture medium therefore already contains some of the compounds. When fungal cells cross the interface with the air, conidiogenone **2** rapidly accumulates at the surface of the hypha, and could reach a threshold concentration which will trigger conidiogenesis. This rapid accumulation can be mimicked in liquid culture by adding **2** to the medium.[10]




**1** **2**

**Figure 1.**

*Keywords*: conidiogenol; conidiogenone; diterpenes; conidiogenesis induction; *Penicillium cyclopium*.

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## 2. Results and discussion

The two diterpenes were isolated by bioassay-guided fractionation of extracts of *P. cyclopium* (prepared according to the experimental part), and obtained in submilligram amounts (from 300 L medium). In short, the ability of the extracts of spent medium, which had supported growth for 48 h, to trigger the induction of conidiation in fresh liquid cultures of *P. cyclopium* was used as the activity assay.[10] High resolution EIMS experiments indicated that the elemental composition of the compounds is $C_{20}H_{34}O_2$ and $C_{20}H_{32}O_2$, respectively, suggesting that the two metabolites are related. The unsaturation index is consequently 4 for conidiogenol **1** and 5 for conidiogenone **2**, and as the 1D NMR data (see Table 1) show that **1** only contains saturated carbons while **2** has a keto function both compounds should have four rings. The structures of **1** and **2** were determined with data obtained from 2D NMR COSY, NOESY, HMQC and HMBC experiments, and the pertinent correlations observed in the HMBC and NOESY experiments with conidiogenol **1** are summarised in Fig. 2.

The HMBC correlations from the methyl protons established that C-16 and C-17 share a neighbouring

**Table 1.** $^1$H (500 MHz) NMR data ($\delta$, multiplicity, $J$) and $^{13}$C (125 MHz) NMR data ($\delta$, multiplicity) for conidiogenol **1** and conidiogenone **2** in $CDCl_3$[a]

| C | Conidiogenol **1** | | Conidiogenone **2** | |
|---|---|---|---|---|
| | $^1$H | $^{13}$C | $^1$H | $^{13}$C |
| 1 | 3.82, dd, 3, 3 | 75.1, d | | 213.8, s |
| 2a | 1.95, m | 39.0, t | 2.75, dd, 5.8, 14.6 | 46.9, t |
| 2b | 1.82, ddd, 3.2, 10.9, 13.8 | – | 2.51, dd, 9.0, 14.6 | – |
| 3 | 3.54, ddd, 4.2, 11, 11 | 69.9, d | 3.50, dddd, 5, 6, 9, 10 | 72.7, d |
| 4 | 1.55, m | 43.6, d | 1.77, qd, 6.5, 10.2 | 44.5, d |
| 5 | – | 61.3, s | – | 64.2, s |
| 6 | 2.38, ddd, 4.6, 9.4, 9.6 | 55.6, d | 2.56, ddd, 4.3, 9, 9 | 54.8, d |
| 7a | 1.94, m | 30.0, t | 1.97, dddd, 3.1, 9, 9, 13.2 | 31.2, t |
| 7b | 1.52, m | – | 1.55, m | – |
| 8a | 2.04, m | 38.6, t | 2.36, ddd, 9, 9, 12.8 | 39.2, t |
| 8b | 1.43, m | – | 1.67, m | – |
| 9 | – | 48.8, s | – | 59.8, s |
| 10a | 1.78, d, 15.2 | 43.3, t | 1.74, d, 15.1 | 43.1, t |
| 10b | 1.77, d, 15.2 | – | 1.52, d, 15.1 | – |
| 11 | – | 54.5, s | – | 54.4, s |
| 12a | 1.72, m | 41.3, t | 1.72, m | 41.4, t |
| 12b | 1.62, m | – | 1.64, m | – |
| 13a | 1.64, m | 40.2, t | 1.65, m | 40.4, t |
| 13b | 1.44, m | – | 1.47, m | – |
| 14 | – | 42.4, s | – | 42.5, s |
| 15 | 1.47, m | 73.1, d | 1.50, m | 72.8, d |
| 16 | 1.10, d, 6.5 | 12.6, q | 1.20, d, 6.5 | 12.8, q |
| 17 | 1.20, s | 23.3, q | 1.22, s | 21.6, q |
| 18 | 1.26, s | 31.0, q | 1.25, s | 30.6, q |
| 19 | 0.98, s | 27.0, q | 1.01, s | 26.9, q |
| 20 | 1.04, s | 31.2, q | 1.04, s | 31.1, q |
| 3-OH | | | 1.60, d, 5.0 | – |

[a] The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

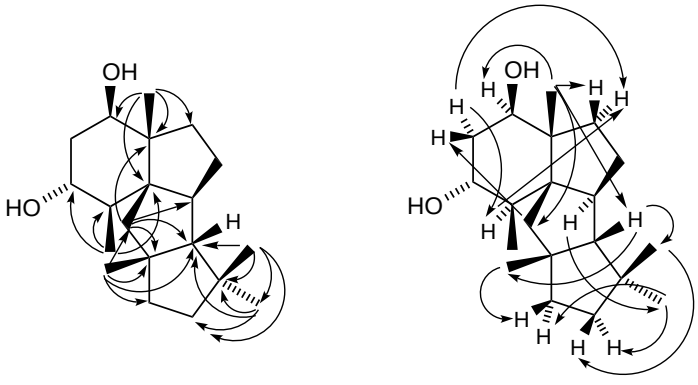


**Figure 2.** Pertinent HMBC (left) and NOESY (right) correlations observed with conidiogenol **1**.

carbon (C-5), as 16-$H_3$ correlate to C-3, C-4 and C-5 while 17-$H_3$ correlate to C-1, C-5, C-8 and C-9. This observation together with the proton spin system from 16-$H_3$–4-H–3-H–2-$H_2$–1-H closes the six-membered ring. 8-$H_2$, which gives HMBC correlations to C-1, C-7, C-9 and C-17, is the starting point of a second proton spin system continuing with 7-$H_2$ and 6-H stopping at 15-H. C-15, with an unusual chemical shift (73.1 ppm), is close to all three remaining methyl groups, as indicated by HMBC correlations (see Fig. 2). Two of these (C-19 and C-20) are, in addition, geminal. The multitude of HMBC correlations from 10-$H_2$ (a few are shown in Fig. 2) do not only close the second ring, by establishing the bond between C-5 and C-6 (also supported by HMBC correlations from 4-H to C-6 and 7-$H_2$ to C-5), but also the third. The two remaining loose ends, C-12 and C-13, both methylene groups, must be connected to each other as one additional ring is required and no more atoms are available. The bond between C-12 and C-13 is also supported by COSY correlations between 12-$H_2$ and 13-$H_2$.

The relative configuration of conidiogenol **1** was determined by examining the correlations in the NOESY spectrum. 17-$H_3$ correlate with 1-H, 8-Hb, 10-H and 15-H, indicating that C-10 and C-17 as well as 15-H are on the same side of the ring system and that the C-9 methyl group is directed towards C-15. The small $^1$H–$^1$H coupling constants of 1-H suggest that it is in an equatorial position, and this is supported by the NOESY correlations from H-1 to both H-2a and H-2b as well as 8-Ha and 8-Hb. With the C-9 methyl group β and pseudoaxial, and the C-1 hydroxyl group β and axial, the six-membered ring adopts a chair-like conformation, in which 2-Hα (=2-Hb) is close in space to 8-Hα. A strong NOESY correlation between 2-Hb and 8-Ha (=8-Hα) confirms this, and NOESY correlations between both these protons and 4-H shows that the C-4 methyl group is β and equatorial. 3-H couples with both 2-Hb and 4-H with $J$ >10 Hz, indicating that it is axial as well, and the NOESY correlation to 10-H confirms that it is β. 6-H gives NOESY correlations to 4-H as well as 16-$H_3$ and 19-$H_3$, and is consequently α, while 15-H correlates with 18-$H_3$ and 20-$H_3$, besides 17-$H_3$, and is β. In agreement with this, both 18-$H_3$ and 20-$H_3$ give NOESY correlations with 12-Hβ (=12Hb) and 13-Hβ (=13-Hb), while 19-$H_3$ correlate to 12-Hα and 13-Hα. The corresponding NMR correlations were observed with conidiogenone **2**, HMBC correlations were now observed between 2-$H_2$, 8-$H_2$ as well as 17-$H_3$, and the keto carbon, showing that it has the same basic structure and relative configuration. Both compounds are novel; diterpenes with the same skeleton to our knowledge have not been reported. We therefore suggest the general name cyclopiane for this type of diterpene.

The identification of conidiogenol **1** and conidiogenone **2** as inducers of conidiogenesis in *P. cyclopium* will provide a new tool for the study of this important morphogenetic event. Although the mechanism by which **2** exerts its triggering effect is not known, it can be envisaged as an interaction with a receptor linked to a signal transduction pathway, and the receptor as well as the pathway could become targets for novel bioactive agents that stimulate or inhibit conidiogenesis in *P. cyclopium*. It is probable that other filamentous fungi have the same or a similar system for the induction of conidiogenesis, such mechanisms have already been found in prokaryotic microorganisms,[11] and this is currently under investigation in our laboratories. Compounds **1** and **2** are additional examples of secondary metabolites with a specific ecological function, in this case to report to the producing organism about the environmental conditions and induce conidiogenesis when the time is right.

## 3. Experimental

### 3.1. Spectroscopy

$^1$H (500 MHz) and $^{13}$C NMR (125 MHz) spectra were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts ($\delta$) are given in ppm, and the coupling constants ($J$) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}=145$ Hz and $^nJ_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra were recorded with a Jeol SX102 spectrometer, while UV spectra were recorded with a Varian Cary 2290 spectrometer. The optical rotations were measured with a Perkin–Elmer 141 polarimeter at 22°C.

### 3.2. Isolation of conidiogenol and conidiogenone

Batch fermenter cultures of *P. cyclopium* Westling (IMI 229034, spore isolate DSS03) were performed as previously described,[12] in 10 L fermenters of F medium (Foster's medium, consisting of 20 g sucrose, 6.0 g $NaNO_3$, 1.5 g $K_2HPO_4$ and 0.5 g $MgSO_4{\cdot}7H_2O$ and a trace element supplement per litre) with an inoculum load of $2.5\times10^6$ conidia mL$^{-1}$ and an aeration rate of 6 L min$^{-1}$ at 25°C. Purification was accomplished from 300 L of 48 h old mF medium (mature F medium, a Foster's medium which has previously supported growth of *P. cyclopium* for 36 h), prepared in 30 independent 10 L-batches. Following filtration (0.45 μm pore size) and adjustment to pH 7.0, each 10 L-batch fermentation filtrate was subjected to solid phase extraction by passing through a 10 g SUPELCLEAN LC-Phenyl column (SUPELCO). After column washing with 50 mL 50% (v/v) aqueous methanol, retained activity was eluted with 150 mL 100% methanol and kept at –20°C. Pooled extracts arising from 300 L mF were finally evaporated to dryness, leaving 595 mg of a brown-yellowish oily residue. This residue was then dissolved in a minimum volume of methanol and fur-

ther purified by reverse phase semipreparative HPLC in a Hypersil-ODS column (10×250 mm, 5 μm particle size) under an acetonitrile/water gradient (starting mobile phase, 45% (v/v) acetonitrile; final mobile phase, 100% acetonitrile; gradient time, 30 min; flow rate, 2.2 mL $min^{-1}$; UV detector, 210 nm). Two major fractions (A and B) with conidiation inducing activity were collected which, after solvent evaporation, yielded two yellowish oily residues of 16.3 and 4.9 mg, respectively. The final purification step was carried out by normal pressure column chromatography on silica gel with diethyl ether. At the end of the purification procedure, 0.70 mg conidiogenol **1** and 0.64 mg conidiogenone **2** were obtained as pure compounds.

### 3.3. Conidiogenol 1

Compound **1** was obtained as a colourless oil. $[\alpha]_D = -20$ (*c* 0.07 in $CHCl_3$). UV (MeOH): no maxima above 210 nm. See Table 1 for $^1H$ and $^{13}C$ NMR data. EIMS (70 eV), *m*/*z* (rel. int.): 306.2566 (1%, $M^+$, $C_{20}H_{34}O_2$ requires 306.2559), 288.2454 (34%, $M^+-H_2O$, $C_{20}H_{32}O$ requires 288.2453), 273 (15%), 267 (50%), 233 (29%), 204 (53%), 203 (100%), 135 (17%), 109 (22%), 95 (15%).

### 3.4. Conidiogenone 2

Compound **2** was obtained as a colourless oil. $[\alpha]_D = -35$ (*c* 0.06 in $CHCl_3$). UV (MeOH): no maxima above 210 nm. See Table 1 for $^1H$ and $^{13}C$ NMR data. EIMS (70 eV), *m*/*z* (rel. int.): 304.2403 (56%, $M^+$, $C_{20}H_{32}O_2$ requires 304.2402), 286.2289 (47%, $M^+-H_2O$, $C_{20}H_{30}O$ requires 286.2296), 271 (13%), 231 (20%), 204 (100%), 203 (59%), 154 (42%), 126 (35%), 109 (22%), 95 (15%).

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