

Notes

SECONDARY METABOLITES BY
CHEMICAL SCREENING. 21[†]CLONOSTACHYDIOL, A NOVEL
ANTHELMINTIC MACRODIOLIDE
FROM THE FUNGUS
Clonostachys cylindrospora
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(Received for publication August 10, 1992)

Application of our chemical screening methodology²⁾ to different new fungal isolates gave rise to the detection, isolation, structure elucidation, and widespread biological testing of a new fungal metabolite named clonostachydiol (1), which is related to the macrodiolides of the colletodioid family^{3,4)}.

The producing organism, strain FH-A 6607, was isolated from a soil sample collected near Rantepao, Sulawesi Island (Indonesia) and exhibited a characteristic brown to yellow colored mycelium. The typical conidiophores were found to be in close morphological analogy to gliocladium and are arranged in self-contained long rows (Fig. 1). Combined with further taxonomical investigations the strain FH-A 6607 was classified as *Clonostachys cylindrospora*⁵⁾. This species belongs to the order of *Moniliales*, which the colletodioid producer *Colletotrichum capsici* is to be integrated into the order of *Melanconiales*, which is more related to the genus *Fusarium*. The structurally related grahamimycin⁶⁾ is produced by *Cytospora* sp. and belongs to a third order of fungi named *Sphaeropsidales*⁷⁾.

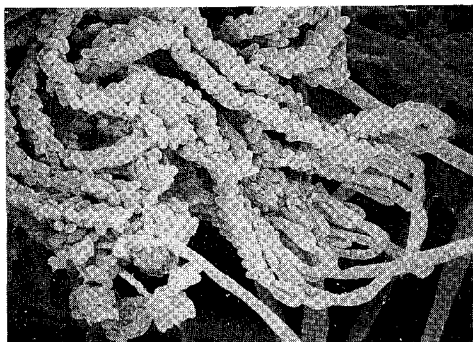
The strain FH-A 6607 was cultivated in a medium containing malt extract 2%, yeast extract 0.2%, glucose 1%, and $(\text{NH}_4)_2\text{HPO}_4$ 0.05% (pH 6.0 prior to sterilization). Production was

carried out in 300-ml Erlenmeyer flasks containing 100 ml of the production medium (200 rpm on a rotary shaker, 25°C) or in 10-liter fermenters (250 rpm; aeration: 4 liters/minute) for 98 hours.

In our screening routine^{2,8)}, we prepared defined extracts of the culture broth from the 100-ml Erlenmeyer flask fermentation followed by a detailed TLC analysis (HPTLC-silica gel 60F₂₅₄ on glass, Merck; see Table 1). By the use of different staining reagents strain FH-A 6607 showed a striking green spot with anisaldehyde- H_2SO_4 (Rf 0.58, CHCl_3 -MeOH, 9:1). Thus, the present study was designed to investigate this metabolite. The culture filtrate (about 9 liters) of a 10-liter fermenter was extracted three times with 3 liters of ethyl acetate. The organic layers were combined, evaporated to dryness and the remaining oily residue was chromatographed on a silica gel column (20 × 4.5 cm, ethyl acetate-*n*-hexane, 1:1). Further purification was performed using Sephadex LH-20 column chromatography (100 × 2.5 cm, MeOH) to obtain 32.7 mg/liter colorless crystalline clonostachydiol (1), which is soluble in MeOH, CHCl_3 , DMSO, and acetone and insoluble in H_2O or *n*-hexane. To receive sufficient material for biological testing, fermentations were carried out in 200-liter scales using the conditions described above. After centrifugation, the culture broth was adjusted to pH 7.0 with 2N NaOH and adsorbed on Amberlite XAD-16. The residue was eluted with

Fig. 1. Scanning electron micrograph of the clonostachydiol producing fungus *Clonostachys cylindrospora*.

The strain has been cultivated on malt extract agar for 10 days at 25°C (strain FH-A 6607; ×1,000).



[†] Part 20: See ref 1.

Table 1. Rf values, color reactions, and physico-chemical properties of clonostachydiol (1).

	Clonostachydiol (1)
MP	164°C
Rf values:	0.58 ^a , 0.30 ^b , 0.95 ^c
Color reactions:	
Anisaldehyde - H ₂ SO ₄	Green
EHRlich's reagent	No colorization
Orcinol reagent	Grey
Blue tetrazolium reagent	Violet
Molecular formula	C ₁₄ H ₂₀ O ₆
MW	284
HRFAB-MS (M ⁺ , m/z) ^d	284.1260
Anal Calcd for C ₁₄ H ₂₀ O ₆	C 59.13 H 7.09
Found	C 59.24 H 6.99
[α] _D ²⁰ (c 1.0, MeOH)	+103°
UV λ _{max} ^{MeOH} nm (ε)	209 (16,400)
IR (KBr) cm ⁻¹	1690, 1652, 1641
CD λ _{extreme} nm ([θ] _D ²⁰)	221 (+85,000)

^a CHCl₃ - MeOH (9:1).

^b EtOAc - *n*-hexane (1:1).

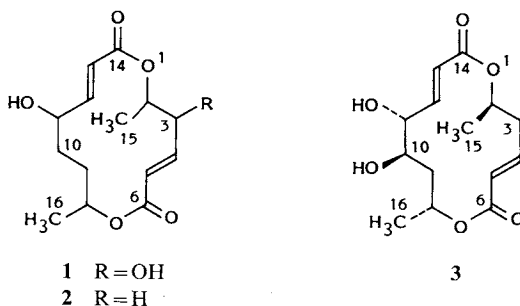
^c BuOH - acetic acid - H₂O (4:1:5, upper phase).

^d Found as calcd.

MeOH - H₂O (4:1). The brownish oily crude evaporation product (308 g) was extracted twice with 1 liter MeOH, the remaining solid (23 g after drying) was extracted twice with 400 ml ethyl acetate, the organic layer was concentrated and **1** crystallized after cooling to 5°C (yield approximately 2.5 g).

The structure of clonostachydiol (**1**) was ascertained by detailed analysis of ¹H, ¹³C and 2D NMR spectra (data see Table 2) based on the molecular formula (C₁₄H₂₀O₆) independently elucidated by both, HRFAB mass spectroscopy and elemental analysis (Table 1). The positions of the substituents in the carbon chains as well as the 14-membered ring resulted unambiguously from the cross peaks in the ¹H-¹H COSY NMR experiment. From the coupling constants it was deduced that the double bonds are *trans*-configured. Furthermore, the location of the double bonds was ascertained from the chemical shifts in the ¹³C NMR spectrum (proton connectivity was deduced from the ¹H-¹³C COSY NMR spectrum) and was consistent with the UV-data. Stereochemical investigations are currently underway. By treatment with acetic anhydride in pyridine (10:1, room temperature, 1 hour) clonostachydiol (**1**) formed a diacetate in nearly quantitative yield (C₁₈H₂₄O₈, EI-MS: m/z 368). The NMR data of the diacetate

Fig. 2. Structures of clonostachydiol (1), colletalol (2), and colletodiol (3).

Table 2. ¹H and ¹³C NMR data of clonostachydiol (1), δ values in ppm, TMS as internal standard, multiplicity assignments by attached proton test (APT), solvent DMSO-*d*₆.

Proton	δ (ppm, <i>J</i> in Hz) ^a	Carbon	δ (ppm) ^b
2-H	5.02 (pseudo-quintet)	C-2	71.4 (d)
3-H	4.18 (m)	C-3	74.8 (d)
4-H	6.75 (dd, <i>J</i> _{3,4} = 6.5, <i>J</i> _{4,5} = 15.8)	C-4	147.4 (d)
5-H	5.97 (dd, <i>J</i> _{5,3} = 1.4)	C-5	123.2 (d)
—	—	C-6	164.6° (s)
8-H	5.16 (m)	C-8	69.1 (d)
9-H ₂	1.45 (m)	C-9	25.7 (t)
10-H ₂	1.58, 1.85 (m)	C-10	28.2 (t)
11-H	4.50 (m)	C-11	68.1 (d)
12-H	6.87 (dd, <i>J</i> _{12,11} = 4.0, <i>J</i> _{12,13} = 15.9)	C-12	152.7 (d)
13-H	5.93 (dd, <i>J</i> _{13,11} = 1.8)	C-13	119.9 (d)
—	—	C-14	164.9° (s)
15-H ₃	1.47 (d, <i>J</i> _{15,2} = 6.5)	C-15	17.4 (q)
16-H ₃	1.22 (t, <i>J</i> _{16,8} = 6.5)	C-16	17.3 (q)
3-OH	2.49 (d, <i>J</i> _{3-OH,3} = 6.8)	—	—

^a 360 MHz.

^b 75 MHz.

^c Signal assignment exchangeable.

are in agreement with the signal shifts to be expected (*e.g.* downfield shift of 3-H/11-H, δ_H (CDCl₃) 5.15/5.54, two additional signals of acetyl groups: δ_H (CDCl₃) 2.06/2.10, δ_C (CD₃OD) 20.7/20.8 and 171.3/171.6). Therefore, **1** was identified as 3,11-dihydroxy-2,8-dimethyl-1,7-dioxacyclotetradeca-4,12-diene-6,14-dione, named clonostachydiol, which structurally has to be integrated into the colletodiol family^{3,4}, from which biosynthetic⁹ and synthetic^{10,11} studies have been reported. However, **1** represents the 3-hydroxy derivative of colletalol (**2**)³.

Clonostachydiol (**1**) was tested in a number of different biological tests. In the basic antibacterial,

antifungal, antiviral, antiprotozoal, herbicidal, and insecticidal assays, each performed with a number of different test organisms^{1,2}, **1** exhibits no significant activity. This is in accordance to colletodiol (**3**), which was described to be biologically inactive. In contrast, we observed a weak cytostatic effect of clonostachydiol (**1**) in proliferation assays (MTT-reduction) with the cell lines L 1210, HT 29, and A 549 (IC_{50} = 4.5, 4.2, and 5.7 μ g/ml, respectively). Based on prior work on the macrodiolide elaiophylin^{1,2} the anthelmintic action of clonostachydiol was investigated in *in vivo* tests using lambs (30 to 40 kg body weight) artificially infected with infective stages of abomasum nematodes (*Haemonchus cortortus*). By the application of 2.5 mg/kg of **1** subcutaneously, a 80 to 90% reduction of the nematodes measured by coproscopic investigations before and after 14 days was found.

Acknowledgment

We thank the Bundesministerium für Forschung und Technologie (BMFT) for financial support (grant 0319311B) and appreciate the excellent technical assistance of A. GODAWA, D. KORNBÜH, M. OSWALD, K. SPITZENBERGER, and P. STAFF. We thank H. KLUGE for valuable discussions and performing additional NMR experiments and D. DÜWEL for anthelmintic testing.

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