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Cylindrocyclin A, a New Cytotoxic Cyclopeptide from *Cylindrocarpon* sp.

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Dedicated to the memory of Professor Kenneth L. Rinehart.

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Abstract In the course of a screening of fungal extracts for new metabolites with cytotoxic activities cylindrocyclin A (**1**) was isolated. The producing strain was identified as *Cylindrocarpon* sp. by microscopy and ITS rDNA sequence analysis. **1** is a novel compound that exhibits cytotoxic acticity against six different cell lines with IC_{50} values ranging from 11 to 53 μM. **1** has no antibacterial or antifungal activity. The compound is a cyclic nonapeptide comprising three alanines, five leucines and one isoleucine. Four amino acids are *N*-methylated. Its structure was elucidated by spectroscopic methods.

Keywords new metabolite, cyclopeptide, cytotoxic, cylindrocarpon

Introduction

Many bacteria and fungi produce low-molecular weight peptides, depsipeptides, peptidolactones, and lipopeptide lactones with linear, branched or cyclic structures as secondary metabolites [1]. They are assembled by large multifunctional enzymes, the nonribosomal peptide synthetases (NRPS). The amino acids can be modified by *e.g. N*-methylation or acetylation. Since the stringency of amino acid recognition by the NRPS is not as high as in ribosomal protein synthesis in many cases a series of closely related cyclopeptides with exchanged amino acids are produced [2]. Many cyclopeptides have interesting biological activities and some of them have been commercialized *e.g.* cyclosporins and echinocandins. In a screening of fungal extracts we found that *Cylindrocarpon* sp. strain A101-96 produced a number of new cytotoxic cyclic nonapeptides. Here we describe the taxonomy of the producing strain, the fermentation, isolation, biological activities and the structure elucidation of the main component that we have named cylindrocyclin A (**1**).

Materials and Methods

Producing Organism

Cylindrocarpon sp. strain A101-96 was isolated from a twig by Prof. H. Anke (IBWF). The material was collected in Alpes Maritimes, France. The fungus was identified by microscopy and ITS sequence analysis as described previously [3]. Although *Cylindrocarpon* sp. strain A101-96 showed the characteristics of the genus, the species could not be unequivocally determined. Voucher specimen and mycelial cultures are deposited in the culture collection of the IBWF e.V., Kaiserslautern. For maintenance on agar slants the fungus was grown in YMG medium (g/liter): yeast extract 4, malt extract 10, glucose 10 and agar 1.4% for solid media. The pH was adjusted to 5.5.

Fermentation

Fermentations were carried out in a Biolafitte C6 fermentor containing 20 liters of YMG medium with aeration (3 liters

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air/minute) and stirring (120 rpm) at room temperature. A well-grown culture (in 250 ml YMG medium in a 500 ml Erlenmeyer flask grown at 22°C and 120 rpm) was used as inoculum. When the glucose was completely consumed (after 2～3 days), the culture fluid was separated from the mycelia by filtration. The culture broth was passed through Mitsubishi Diaion HP21 resin and washed with 1.5 liters of H_2O/MeOH (1 : 1). The active components were eluted with MeOH and the organic phase concentrated *in vacuo*.

Isolation of the Compounds

The crude extract of the methanol fraction (see above, 541 mg) was applied onto a column (2.5×10 cm) containing silica gel (Merck 60, 0.063～0.2 mm). An enriched product (96 mg) was obtained after elution with ethyl acetate/MeOH (3 : 1). Preparative HPLC (Merck Lichrosorb® RP 18, 7 μm; column 25×250 mm; flow 15 ml/minute; gradients: H_2O - MeCN 50～75% in 10 minutes, 75～100% in 15 minutes, 10 minutes at 100%) yielded 10 mg of an enriched product (Rt=30～33 minutes). This was separated by analytical HPLC (Zorbax Eclipse XDB Phenyl 4.6×150 mm, 5 μm, Fa. Agilent; Flow: 2.5 ml/minute; isocratic: 55% MeCN; temperature: 30°C) and yielded 7 mg of **1** (Rt=6.07 minutes).

Spectroscopy

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe 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 (δ) 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). LC-MS spectra were recorded with a HP 1100; APCI, positive/negative mode, while the HRESIMS spectrum was recorded with a Micromass Q-TOF MICRO instrument with the resolution 5000 in positive ion mode. The instrument was calibrated with phosphoric acid (0.1% in water : acetonitrile 5 : 5) and the reference ion at 980.7768 was used as lock mass. The IR spectra were recorded with a Bruker IFS 48 spectrometer, the melting point (uncorrected) were determined with a Reichert microscope, and the optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22°C.

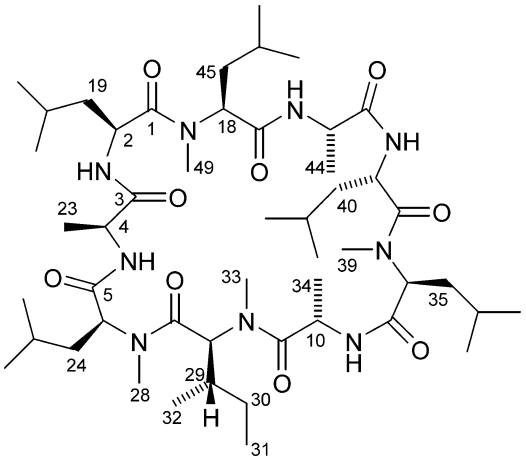

Fig. 1 Structure of cylindrocyclin A (**1**).

Cylindrocyclin A (1)

1 was obtained as a white powder, mp 220°C (decomp.). $[\alpha]_D^{22}$ −5° (*c* 0.5 in DMSO). IR (KBr) 3297, 2959, 1636, 1533, and 1086 cm^{-1}. ^{1}H and ^{13}C NMR data are given in Table 1. HRMS (ESI, M+H$^+$) found *m/z* 948.6851, $C_{49}H_{90}N_9O_9$ requires 948.6862. The L-configuration of the amino acids contained in cylindrocyclin A was determined after hydrolysis using Marfey's reagent and HPLC analysis of the diastereomers (column: Zorbax eclipse XDB Phenyl 3.5 μm, 3×150 mm; 65% water containing 0.1% formic acid, 35% acetonitrile; 0.6 ml/minute) according to [4]. The retention times observed were for L-Ala: 2.7, L-Leu: 6.4, NMe-L-Leu: 7.8 and NMe-L-Ile: 8.3 minutes. They were identical with authentical derivatives of L-Ala, L-Leu, NMe-L-Leu and NMe-L-Ile prepared the same way.

Biological Assays

Antimicrobial activities were determined in the agar plate diffusion assay as described previously [5]. Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested as described by Anke *et al.* [5]. Nematicidal activity was measured as described previously [6].

Colo-320 cells (DSMZ ACC 144, human colo adenocarcinoma), Jurkat cells (ATCC TIB 152, human acute T cell leukemia), L1210 (ATCC CCI 219), and HL-60 cells (DSMZ ACC 3, human promyelocytic leukemia) were grown in RPMI 1640 medium (GIBCO, BRL), MDA-MB-231 (ATCC HTB26) cells and MCF-7 cells in D-MEM medium (GIBCO, BRL). The medium was supplemented with 10% fetal calf serum (FCS) (GIBCO, BRL), 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate. The cells were maintained in a humidified atmosphere containing 5% of CO_2 at 37°C.

Cytotoxicity was measured in microtiter plates with $1\sim5\times10^5$ cells/ml medium. Cells were incubated with and without test compounds. In addition to a microscopic examination after 24, 48, and 72 hours, the effect on the growth of monolayer cell lines was measured by Giemsa staining. The viability of suspension cells was measured in a test based on XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide).

Results and Discussion

Both the typical morphological characteristics and the ITS sequence were in full accordance with the genus *Cylindrocarpon*. The sequence identity to *Cylindrocarpon faginatum* (CBS 217.67) AY677277.1 was 98% and to *Neonectria ramulariae* AJ279446.1 97%. *Nectria* and *Neonectria* are teleomorphs of the anamorph *Cylindrocarpon*. A number of cyclosporins have been obtained from *Cylindrocarpon* [7]. **1** was obtained as described in the experimental section. High resolution mass spectrometry suggested that its elemental composition was $C_{49}H_{89}N_9O_9$, and this was supported by the 1D NMR spectra. **1** consequently had ten unsaturations, and the presence of only nine signals for unsaturated carbons, all as carbonyl groups, in the ^{13}C NMR spectrum indicated that the compound contained one ring. Further analysis of the 1D NMR data (shown in Table 1) clearly suggested that **1** was made up from nine amino acids, including four that were *N*-methylated. Careful analysis of the COSY spectrum, confirmed by the HMBC data, revealed that there were two leucines, three *N*-methylleucines, three alanines and one *N*-methylisoleucine. Because all five of the non-methylated nitrogens were observed to be protonated **1** was deduced to be a cyclic nonapeptide. To establish the structure, the COSY correlations between the NH as well as the N–CH_3 protons and the α-protons were established (long range ^1H-^1H couplings between the *N*-methyl protons and the α-methine protons were clearly visible in a high resolution COSY experiment), and the HMBC correlations between the same protons as well as the α- and β-protons and the carbonyl carbons. Confirmation was obtained from the HMBC correlations between the NH, the N–CH_3 and the β-protons and the α-carbons. Starting with the NH:s, COSY correlations from 2-NH *via* 2-H, 19-H_2 and 20-H to 21-H_3 and 22-H_3, from 4-NH *via* 4-H to 23-H_3, from 10-NH *via* 10-H to 34-H_3, from 14-NH *via* 14-H, 40-H_2 and 41-H to 42-H_3 and 43-H_3, and from 16-NH *via* 16-H to 44-H_3, established the obvious proton spin systems in three alanines and two leucines. The COSY correlations from 28-H_3 *via* 6-H, 24-H_2 and 25-H to 26-H_3 and 27-H_3, from 33-H_3 *via* 8-H and 29-H to 32-H_3 as well as *via* 8-H, 29-H and 30-H_2 to 31-H_3, from 39-H_3 *via* 12-H, 35-H_2 and 36-H to 37-H_3 and 38-H_3, and from 49-H_3 *via* 18-H, 45-H_2 and 46-H to 47-H_3 and 48-H_3, established the proton spin systems in remaining three *N*-methylleucines and the *N*-methylisoleucine. All NH's as well as the *N*-methyl protons gave HMBC correlations to both the neighboring α-carbon and the carbonyl carbon. Consequently, 2-NH gave HMBC correlations to C-2 and C-3, 4-NH gave HMBC correlations to C-4 and C-5, 28-H_3 gave HMBC correlations to C-6 and C-7, 33-H_3 gave HMBC correlations to C-8 and C-9, 10-NH gave HMBC correlations to C-10 and C-11, 39-H_3 gave HMBC correlations to C-12 and C-13, 14-NH gave HMBC

Table 1 ^1H (500 MHz) (δ; multiplicity; *J*) and ^{13}C (125 MHz) (δ; multiplicity) NMR data for **1**

C	^1H	^{13}C	C	^1H	^{13}C
1	—	172.7; s	28	2.84; s	28.7; q
2	4.85; m	48.8; d	29	2.26; m	33.2; d
3	—	173.1; s	30	1.47/1.13; m	25.4; t
4	4.86; m	47.8; d	31	0.90; t; 8.1	9.4; q
5	—	168.2; s	32	0.94; d; 6.6	16.0; q
6	4.91; m	58.6; d	33	3.23; s	31.0; q
7	—	170.4; s	34	1.13; d; 6.6	18.6; q
8	5.20; d; 11.2	55.1; d	35	1.75/1.58; m	34.8; t
9	—	173.8; s	36	1.48; m	24.8; d
10	4.84; m	45.6; d	37	0.93; d; 6.7	22.7; q
11	—	168.5; s	38	0.88; d; 6.7	22.5; q
12	4.92; m	53.7; d	39	2.88; s	29.7; q
13	—	173.6; s	40	1.31/1.08; m	43.6; t
14	4.94; m	47.5; d	41	1.65; m	24.6; d
15	—	172.9; s	42	1.04; d; 6.7	23.8; q
16	4.48; m	51.7; d	43	0.79; d; 6.7	23.5; q
17	—	169.5; s	44	1.48; d; 7.3	18.4; q
18	4.45; m	59.1; d	45	2.21/1.50; m	37.4; t
19	1.75/1.47; m	40.2; t	46	1.60; m	24.7; d
20	1.89; m	25.5; d	47	0.95; d; 6.6	23.1; q
21	1.07; d; 6.4	21.5; q	48	0.95; d; 6.6	23.1; q
22	1.01; d; 6.7	23.6; q	49	2.80; s	29.4; q
23	1.26; d; 7.0	17.7; q	2-NH	6.75; d; 5.0	
24	2.52/1.08; m	38.3; t	4-NH	8.18; d; 9.4	
25	1.67; m	25.4; d	10-NH	7.24; d; 8.3	
26	1.04; d; 6.7	21.5; q	14-NH	7.57; d; 8.7	
27	1.04; d; 6.7	21.3; q	16-NH	8.12; d; 8.7	

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 (δ) are given in ppm and the coupling constants *J* are given in Hz. The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

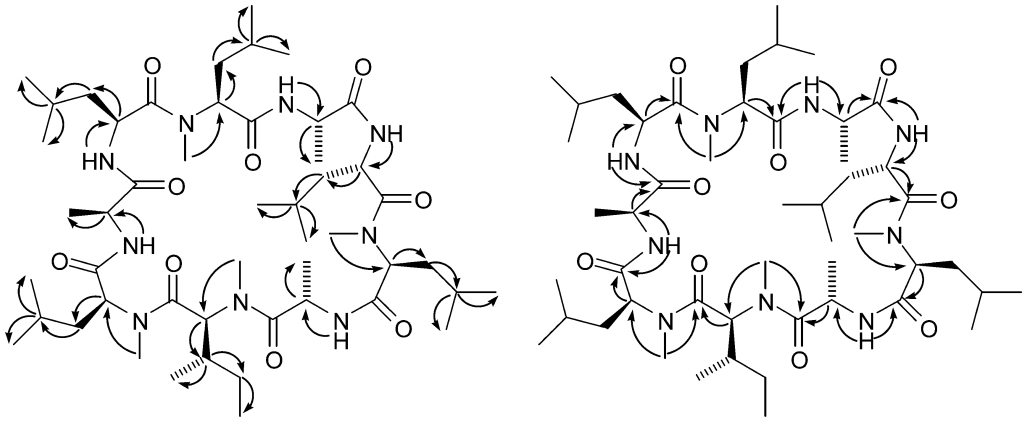

Fig. 2 Pertinent COSY (left) and HMBC (right) correlations observed with **1**.

correlations to C-14 and C-15, 16-NH gave HMBC correlations to C-16 and C-17, and 49-H_3 gave HMBC correlations to C-18 and C-1. In addition, all α-protons gave HMBC correlations to their corresponding carbonyl carbon. 2-H gave a HMBC correlation to C-1, 4-H gave a HMBC correlation to C-3, 6-H gave a HMBC correlation to C-5, 8-H gave a HMBC correlation to C-7, 10-H gave a HMBC correlation to C-9, 12-H gave a HMBC correlation to C-11, 14-H gave a HMBC correlation to C-13, 16-H gave a HMBC correlation to C-15, and 18-H gave a HMBC correlation to C-17. All pertinent COSY and HMBC correlations are summarized in Figure 2. In this way it was possible to establish all peptide bonds unequivocally, and thereby the structure of **1**. To determine the absolute configuration of the compound, it was subjected to acidic hydrolysis to produce the individual amino acids that were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, according to the procedure reported in reference [4]. The hydrolysis mixture was analyzed by the LC-MS methods described above and comparing peaks with D- and L-amino acids standards we determined that all nine amino acids in **1** were of the L configuration. The structure shown in Figure 1 consequently represents the absolute configuration of **1**. In addition, our strain was found to produce the known ilicicolins C, D, E, and F (data not shown), although no cyclosporins or roridins could be isolated [8, 9].

The cytotoxic activities of **1** against human and mouse cell lines are shown in Table 2. The compound did not exhibit either antimicrobial nor nematicidal activities up to concentrations of 100 μg/paper disk or 100 μg/ml respectively. No phytotoxic effects against *Setaria italica* and *Lepidium sativum* were found at 500 μg/ml.

Table 2 Cytotoxic activities of **1**

Cell line	IC_{50} (μM)
COLO-320	11
HL-60	11
L1210	11
Jurkat	11
MDA-MB-231	42
MCF-7	53

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