J. Antibiot. 58(2): 141–144, 2005

NOTE

THE JOURNAL OF ANTIBIOTICS

Bioactive Cyclic Peptides from the Psychrotolerant Fungus *Penicillium algidum*

Petur W. Dalsgaard, Thomas O. Larsen, Carsten Christophersen

Received: October 28, 2004 / Accepted: December 17, 2004

Abstract A new cyclic nitropeptide, psychrophilin D (**1**), together with two known cyclic peptides, cycloaspeptide A (**2**) and cycloaspeptide D (**3**), were isolated from the psychrotolerant fungus *Penicillium algidum* using C_{18} flash chromatography, LH-20 Sephadex and preparative HPLC. The structure of psychrophilin D (**1**) was derived from mass spectrometric information, 1D and 2D NMR spectra and Marfey's method.

The compounds were tested in antimicrobial, antiviral, anticancer and antiplasmodial assays. Psychrophilin D (**1**) exhibited a moderate activity (ID_{50} 10.1 μg/ml) in the P388 murine leukaemia cell assay. Cycloaspeptide A (**2**) and D (**3**) exhibited moderate activity (IC_{50} 3.5 and 4.7 μg/ml, respectively) against *Plasmodium falciparum*.

Keywords *Penicillium algidum*, psychrophilin, cycloaspeptide, P388, antimalaria

During our search for new compounds through UV guided analysis of crude extracts, mainly within the genera *Penicillium* and *Aspergillus*, we recently uncovered the unusual cyclic nitropeptide, psychrophilin A and two other cyclic peptides, cycloaspeptides A (**2**) and D (**3**) [1]. We now wish to report the isolation and structure elucidation of a new cyclic nitropeptide psychrophilin D (**1**). In addition we describe the preliminary *in vitro* testing for antimicrobial, antiviral, anticancer and antiplasmodial activity of psychrophilin D (**1**), cycloaspeptides A (**2**) and D (**3**) (Fig. 1) isolated from the undescribed psychrotolerant fungus Specie Novum *Penicillium algidum* Frisvad.

P. algidum was collected from soil under a *Ribes* sp. east of Oksestien, Zackenberg, Greenland (August 1999). A voucher specimen is retained at the Technical University of Denmark as IBT 22067. An analytical HPLC-DAD analysis showed that the fungus produces a compound with a UV-spectrum similar to that of psychrophilin A [1], but with different retention index [2, 3], on CYA (Czapek yeast autolysate agar contains $NaNO_3$ 3 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$, yeast extract 5 g, sucrose 30 g, agar 20 g and 1 liter distilled water, final pH 6.0~6.5). The fungus was cultivated as tree point mass inoculation on 200 Petri dishes containing CYA at 20°C for 14 days in the dark. Mycelium and agar from the 200 Petri dishes were harvested and extracted twice overnight with EtOAc. After filtration through a Whatman 1PS phase separation filter, the extract was concentrated *in vacuo* to give 1.2 g of crude material. The crude extract was mixed with 1.2 g of celite and directly fractionated using a 10 g C18 Solid Phase Extraction cartridge. Six 100 ml fractions were collected ($MeOH/H_2O$: (10 : 90), (25 : 75), (50 : 50), (75 : 25), MeOH and finally MeOH + 50 μg/ml TFA). The MeOH - water (75 : 25) and MeOH fraction were combined yielding 860 mg of extract after evaporation. This material was further fractionated on a 25×750 mm Sephadex column using LH-20 with MeOH as mobile phase. Ten fractions were collected. The fifth fraction (480 mg) contained psychrophilin D (**1**), cycloaspeptide

C. Christophersen (Corresponding author)**, P. W. Dalsgaard:** Marine Chemistry Section, Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark, E-mail: carsten@kiku.dk

T. O. Larsen: Center for Microbial Biotechnology, BioCentrum-DTU, Building 221, Technical University of Denmark, DK-2800 Lyngby, Denmark

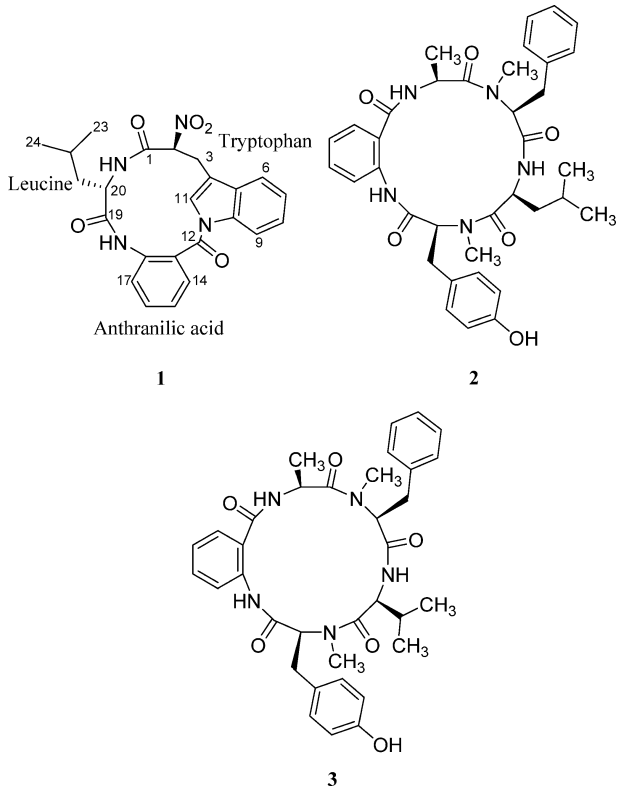

Fig. 1 Psychrophilin D (**1**), cycloaspeptides A (**2**) and D (**3**) isolated from *Penicillium algidum*.

A (**2**), cycloaspeptide D (**3**), griseofulvin and dechlorogriseofulvin, and was purified by HPLC on a preparative Waters Delta Pak C18 (15 μm, 100 Å, 300×19 mm) column (flow rate 30 ml/minute, gradient 50∼75% MeCN+50 μg/ml TFA in 30 minutes) to afford pure **1** (7.4 mg), cycloaspeptide A (**2**) (30 mg), and cycloaspeptide D (**3**) (92 mg). Griseofulvin and dechlorogriseofulvin were also isolated and the identities were confirmed by ESI-MS upon comparison with the authentic compounds.

HRESIMS, ^{13}C-NMR, ^{1}H-NMR and HMQC data for **1** revealed it to have the molecular formula $C_{24}H_{24}N_4O_5$ (15 unsaturation sites) and to possess two exchangeable protons. The ^{1}H- and ^{13}C-NMR data (Table 1) indicated the presence of one *o*-substituted phenyl and one indole moiety. The aliphatic region exhibited two independent spin systems. A X–CH–CH_2–X′ spin system was attached to the indole moiety as shown by HMBC connectivities (Table 1). Analogously, a X–CH–CH_2–$CH(CH_3)_2$ spin system was shown by COSY connectivities. Analysis of the combined data suggested that compound **1** is closely related to the cyclic nitropeptide psychrophilin A [1], which was previously isolated from the psychrotolerant *P. ribeum* [4]. The NMR data for compound **1** show similarity with the set of data reported for psychrophilin A [1], except for the replacement of the proline moiety in psychrophilin A with a leucine group in **1**. The presence of leucine was further substantiated by the signals of N–H doublet at δ 8.32, the α-proton at δ 4.51, the multiplets at δ 1.31∼1.45 and the two methyl doublets at δ 0.79 and δ 0.86. Analysis of COSY, ROESY and HMBC data confirmed the structure as assigned in **1**. The relatively downfield shift of the α-proton signal in tryptophan (δ 5.33) suggested the presence of a nitro group on the α-carbon. This was confirmed by the strong absorptions at 1553 and 1365 cm^{-1} in the IR-spectrum.

The amino acids sequence was deduced from the HMBC and NOE connectivites. The HMBC correlation between 18-NH and carbon 20 established the connection between the anthranilic acid and leucine moieties. A NOE coupling between 20-NH and H-2 connects the aliphatic end of tryptophan and leucine.

The absolute stereochemistry of **1** was determined by hydrolysis and HPLC comparison of the Marfey's [5] derivative with standards derived from authentic *R*- and *S*-leucine. This revealed the leucine moiety to have the *S*-configuration. The stereochemistry around carbon 2 in the tryptophan skeleton was assigned by the method described for psychrophilins B and C [6]. Two 3D models of **1**, (2*S*, 20*S*) and (2*R*, 20*S*), were simulated with minimal energy conformation and the NOE correlation between the α-proton in tryptophan (H-2) and H-3b, H-6 and 20-NH. The proton H-3b shows a strong enhancement to H-6. Proton H-3a shows a weak enhancement to H-11 as well as H-6. Accordingly, we assign the absolute configuration of carbon 2 as *S*. The CD spectra of **1** and psychrophilin A, with established absolute configuration, are qualitatively identical. Psychrophilin D (**1**) has accordingly (2*S*, 20*S*) configuration.

Psychrophilin D (**1**), cycloaspeptides A (**2**) and D (**3**) were tested in antimicrobial, antiviral, anticancer and antiplasmodial assays. In the antimicrobial assay three bacteria (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*) and three fungi (*Candida albicans*, *Trichophyton mentagrophytes*, *Cladosporium resinae*) were used. The paper disk assay (40 μl of a 1 mg/ml solution of **1**, **2** and **3**) did not show any inhibition in these assays. In the antiviral assay **1**, **2** and **3** were tested against *Herpes simplex* type 1 virus (ATCC VR 733) and *Polio* virus type 1 (Pfizer vaccine strain) in infected African green monkey kidney cells (BSC-1). The paper disk assay (40 μl of a 1 mg/ml solution of **1**, **2** and **3**) did not show any inhibition of the viruses or alteration of the host cells. In a P388 murine leukemia cell assay **2** and **3** showed an ID_{50} value higher than 12.5 μg/ml and accordingly were considered inactive. Psychrophilin D (**1**) exhibited an ID_{50} value of

Table 1 NMR data for psychrophilin D (**1**) (400 MHz (^{1}H), 100.6 MHz (^{13}C) in DMSO-d_6. Reference: DMSO-d_6 ^{1}H 2.5 ppm, ^{13}C 39.6 ppm

Psychrophilin D (**1**)				
Position	δ_C	δ_H (mult; *J*, Hz)	HMBC	ROESY
1	164.3			
2	85.8	5.33 (dd; 11.6, 3.9)	C1	3b, 6, 20-NH
3a	25.5	3.30 (m)[a],	C2, C4, C5, C11	11, 6
3b		3.62 (dd; 12.3, 3.9)	C2	2, 6
4	114.0			
5	129.6			
6	119.5	7.84 (d; 7.3)	C8, C10	2, 3b, 3a
7	123.7	7.36 (m)[b]	C5, C9	
8	125.2	7.36 (m)[b]	C10	
9	116.5	8.48 (d; 7.5)		
10	135.1			
11	125.0	6.85 (s)	C4, C5, C10,	3a
12	166.5			
13	—			
14	132.2	7.70 (d; 7.9)	C18	
15	125.3	7.36 (m)[b]	C16, C17	
16	126.0	7.63 (t; 7.8)	C18	
17	122.4	7.54 (d; 7.8)	C16	20
18	133.4			
18-NH		10.22 (s)		20
19	167.9			
20	52.1	4.51 (m)		18-NH, 23, 24, 17, 21
20-NH		8.32 (d; 7.6)		2
21	36.8	1.31 (m)[b], 1.31 (m)[b]		20
22	24.2	1.45 (m)[b]		23, 24
23	22.0	0.79 (d; 7.6)	C21	20, 22
24	22.3	0.86 (d; 6.5)	C21	20, 22

[a] Water signal interfering. [b] Signals are overlapping.

10.1 μ/ml and is thus moderately active. In the antiplasmodial testing psychrophilin D (**1**), cycloaspeptides A (**2**) and D (**3**) were screened against a chloroquine sensitive strain of *Plasmodium falciparum* (3D7). Psychrophilin D (**1**) was inactive, while cycloaspeptides A (**2**) and D (**3**) had a IC_{50} value of 3.5 and 4.7 μg/ml, respectively. This activity is considered as moderate. Chloroquin had a IC_{50} value of 11.8 ng/ml in the same assay.

Experimental

The circular dichroism (CD) spectrum was measured on a modified JASCO 710 instrument. The UV spectra were recorded on a Perkin-Elmer UV/VIS lambda 2 spectrophotometer. Rotation were measured with a Perkin-Elmer 241 polarimeter. IR spectra were measured on a Perkin-Elmer 1760X FT-IR spectrometer. NMR data were recorded in DMSO-d_6 on a Varian 400 FT-NMR spectrometer operating at 400.0 MHz and 100.6 MHz for ^{1}H- and ^{13}C-NMR spectra, respectively. The analytical HPLC data were obtained on Agilent 1100 HPLC-system using Chemstation software and a Hewlett Packard Hypersil BDS-C18, 3 μm, 4.0×100 mm column; flow 1 ml/minute. HRESIMS analyses were performed using a LCT mass spectrometer (Micromass, Manchester, UK). Data were acquired and processed using the MassLynx program. Preparative HPLC was carried out on a Waters 600E system with a Waters 996 Photodiode Array Detector using Millennium software and a Waters Delta Pak C18 column (19 mm×300 mm, 15 μm 100 Å). Marfey's reagent

[N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide] and *R*- and *S*-leucine were purchased from Sigma.

Psychrophilin D (1)

White powder from MeCN/H_2O: mp 93~95°C; $[\alpha]_D^{25}$ +24.4° (*c* 0.18, MeCN); CD λ ext (*c* 0.016, MeCN) ($\Delta\varepsilon$) 201.5 (38.1), 229.5 (−22.9), 239 (−19.2), 275 (4.4), 288 (6.2), 313.5 (−1.7) nm; UV (MeCN) λ_{max} (log ε) 194 (4.52), 244 (4.13), 303 (3.76); IR (KBr) ν_{max} 1365, 1553 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC and ROESY, see Table 1; HREISMS obsd $(M+H)^+$ at *m*/*z* 449.1819, calcd for $C_{24}H_{25}N_4O_5$ 449.1825.

Hydrolysis of psychrophilin D (**1**). The peptide (**1**) (200 μg) was treated at 155°C for 60 minutes with 6 N HCl. After cooling, the sample was freeze-dried and derivatized with Marfey's reagent [5]. The configuration of leucine was determined by using a gradient of H_2O (0.1% TFA)/MeCN (0.1% TFA) (start, 90 : 10; end, 50 : 50) for 40 minutes. Retention times (in minute) for the standards were leucine, *S*, 23.0, *R*, 27.7. The analysis gave the following retention times (in minute): 23.0, establishing the *S*-configuration for the leucine residue.

Acknowledgments This project was supported by the Danish Technical Research Council under the project "Functional biodiversity in *Penicillium* and *Aspergillus*" (grant no. 9901295). Thanks are due to S. E. Harnung for determination of the CD data on an instrument financed by the Danish Natural Science Research Counsil, grant No.//-0373-1. We are indebted to Mr. A. Lynge at Dansk Polar Center for the soil samples, Mrs. E. K. Lyhne and Prof. J. C. Frisvad for taxonomical studies of the fungus, Mrs. G. Ellis for the bioactivity testing, Mrs. D. Brix for the antimalaria testing and Mrs. H. Jacobsen for support with the analytical HPLC and the LC MS analysis.

References

1. Dalsgaard PW, Larsen TO, Frydenvang K, Christophersen C. Psychrophilin A and cycloaspeptide D, novel cyclic peptides from the psychrotolerant fungus *Penicillium ribeum*. J Nat Prod 67: 878–881 (2004)
2. Frisvad JC, Thrane U. Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). J Chromatogr 404: 195–214, 1987
3. Nielsen KF, Smedsgaard J. Fungal metabolites screeining: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography-UV-mass spectrometry methodology. J. Chromatogr A 1002: 111–136, 2003
4. Frisvad JC, Overy DP, Dalsgaard PW, Lyhne EK, Jarvis BB, Fettinger JC, Larsen TO. Four psychrotolerant species with high chemical diversity consistently producing cycloaspeptide A, *Penicillium jamesonlandense* sp. nov., *P. ribeum* sp. nov., *P. soppii* and *P. lanosum*. Int J Syst Evol Micr Submitted.
5. Marfey P. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-Difluoro-2,4-dinitrobenzene. Carlsberg Res Commun 49: 591–596, 1984
6. Dalsgaard PW, Blunt JW, Munro MHG, Larsen TO, Christophersen C. Psychrophilin B and C: cyclic nitropeptides from the psychrotolerant fungus *Penicillium rivulum*. J Nat Prod 67: 1950–1952, 2004