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NOTE

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Bioactive Cyclic Peptides from the Psychrotolerant Fungus *Penicillium algidum*

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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

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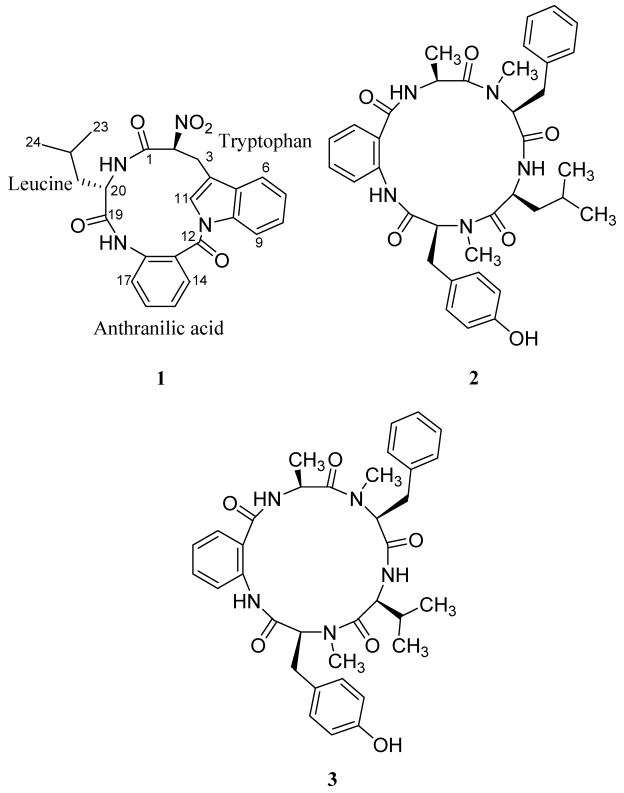

Fig. 1 Psychrophilin D (**1**), cycloaspeptides A (**2**) and D (**3**) isolated from *Penicillium algidum*.

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.

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