Psychrophilin B and C: Cyclic Nitropeptides from the Psychrotolerant Fungus *Penicillium rivulum*

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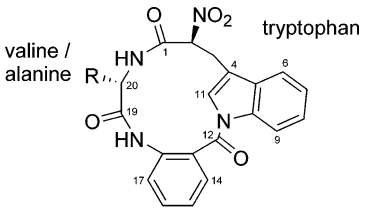
Psychrophilins B (**1**) and C (**2**), two new cyclic nitropeptides, have been isolated from the psychrotolerant, new species *Penicillium rivulum* Frisvad. The nitropeptides were isolated by high-speed countercurrent chromatography (HSCCC) and HPLC using UV-guided fractionation of the organic extract.

The psychrophilic and psychrotolerant fungi from *Penicillium* and *Aspergillus* are chemically unexplored in the primary literature. In our ongoing search for new metabolites as chemotaxonomic markers and for bioactivity studies, we have established that this extremophilic niche is a good source for cyclic peptides[1] and other interesting compounds. Using established dereplication procedures[2] and a UV-guided high-speed countercurrent chromatography (HSCCC) isolation technique afforded two new cyclic peptides, psychrophilins B (**1**) and C (**2**), from the psychrotolerant, new species *P. rivulum* Frisvad. Structures for **1** and **2** were elucidated by using NMR, 2D NMR, HRESIMS, and chemical analysis.



1 R = $CH(CH_3)_2$
2 R = CH_3

The isolate of *P. rivulum* was cultured on CYA-coated LECA nuts.[3] Microscale fractionation of an aliquot of the ethyl acetate extract using a UV-guided HSCCC isolation procedure established a method that was scaled up to a preparative level. Two of the fractions from HSCCC were then further purified by preparative reverse-phase HPLC to afford compounds **1** and **2**.

Psychrophilin B (**1**) was isolated as a white amorphous powder. HRESIMS, ^{13}C NMR, ^{1}H NMR, COSY, and HMQC-DEPT data for **1** established the molecular formula as $C_{23}H_{22}N_4O_5$ (15 degrees of unsaturation), the presence of two exchangeable protons, an *o*-disubstituted benzene, an indole moiety, and two independent aliphatic spin systems. The first, an X−CH−CH_2−X spin system, was attached to the indole moiety as shown by CIGAR connectivities (Table 1). An X−CH−$CH(CH_3)_2$ spin system was also defined. Linking the COSY, CIGAR, and chemical shift data (NH doublet, δ 8.47; α-proton, δ 4.19; CH multiplet, δ 1.86; and two CH_3 doublets, δ 0.86 and 0.92) established that this spin system was part of a valine moiety. These data suggested that **1** was closely related to the known cyclic nitropeptide psychrophilin A,[1] isolated from the psychrotolerant *P. ribeum*. The NMR data for **1** were similar to the partial set of data reported for psychrophilin A[1] except that the proline in psychrophilin A had been replaced by a valine in **1**. The absorption at 1551 and 1366 cm^{-1} in the IR spectrum and the relatively downfield shift (δ 5.46) of H-2 in the ^{1}H NMR spectrum suggested the presence of a nitro group on the α-carbon in tryptophan.

Psychrophilin C (**2**) was isolated as a white amorphous powder. HRESIMS, ^{13}C NMR, ^{1}H NMR, and HMQC-DEPT data for **2** established the molecular formula $C_{21}H_{18}N_4O_5$. In addition **2** and **1** have similar NMR, UV, and IR spectral characteristics. The valine in **1** was replaced by an alanine residue. These deductions were confirmed by the mass difference of 28 between the two compounds.

To address the absolute stereochemistry of **1** and **2**, a sample of each compound was hydrolyzed and chirality of the valine in **1** and alanine in **2** was established as *S* using Marfey's method.[4] The chirality of C-2 cannot be accessed directly because the nitrotryptophan is destroyed during acid hydrolysis. Instead the NOE cross-peaks observed in **1** and **2** were used to determine the most likely configuration (Figure 1). Modeling (2*S*,20*S*) and (2*R*,20*S*) minimal energy variations of psychrophilin C (**2**), based on the crystal structure data from psychrophilin A, revealed the (2*S*,20*S*) configuration in **1** and **2** to be more likely. In this model the amide bonds between anthranilic acid−alanine and alanine−tryptophan were assumed to be *trans*. This indicated the absolute configuration at C-2 as *S* and hence (2*S*,20*S*) for **1** and **2**.

Finally, in support of the (2*S*,20*S*) stereochemical assignments of **1** and **2**, the CD spectra of the compounds were superimposable on the CD spectra recorded for psychrophilin A[1] with defined (2*S*,20*S*) chirality.

Experimental Section

General Experimental Procedures. The circular dichroism (CD) spectra were measured on a modified JASCO 710 spectropolarimeter. The UV spectra were recorded on a Perkin-Elmer UV/vis lambda 2 spectrophotometer. Rotations were measured with a Perkin-Elmer 241 polarimeter. IR spectra were measured on a Shimadzu FTIR-8201 PC spectrometer. ^{1}H and 2D NMR ($^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ HSQC-DEPT, $^{1}H-^{13}C$ CIGAR) spectra were recorded on a Varian INOVA 500

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Table 1. NMR Data for Psychrophilins B (**1**) and C (**2**) (500 MHz (^{1}H) and 75 MHz (^{13}C) in DMSO-d_6)[a]

	psychrophilin B (**1**)				psychrophilin C (**2**)			
atom no.	C_δ	H_δ	mult., *J* (Hz)	CIGAR (H_δ to C_δ)	C_δ	H_δ	mult., *J* (Hz)	CIGAR (H_δ to C_δ)
1	164.7				164.3			
2	85.7	5.46	dd; 11.5, 3.5	C1	85.5	5.45	dd; 11.7, 4.1	C1
3a	25.3	3.40	m[b]	C1, C2, C4, C5, C11	25.2	3.40	m[b]	C1, C2, C4, C5, C11
3b		3.73	dd; 13.0, 3.5	C1, C2, C4, C5, C11		3.72	dd; 12.7, 4.1	C1, C2, C4, C5, C11
4	114.2				113.9			
5	129.7				129.5			
6	119.5	7.95	d; 7.5	C4, C8, C10	119.3	7.96	d; 7.5	C4, C8, C10
7	123.7	7.47	m[c]	C5, C9	123.6	7.45	m[c]	C5, C9
8	125.2	7.47	m[c]	C6, C10	124.9	7.48	m[c]	C6, C10
9	116.5	8.57	br s		116.3	8.60	m	
10	135.2				135.4			
11	123.6	6.94	br s	C4, C5, C10	125.3	6.96	s	C4, C5, C10, C12
12	166.8				166.5			
13	126.5				126.6			
14	132.1	7.79	d; 7.5	C12, C16, C18	132.0	7.81	d; 7.5	C12, C16, C18
15	125.0	7.47	m[c]	C13	124.9	7.48	m[c]	
16	132.0	7.72	t; 7.5	C14, C18	132.1	7.74	m[c]	C14, C18
17	122.2	7.58	d; 7.5	C13, C15	122.3	7.74	m[c]	C12, C15
18	133.4				133.4			
18NH		10.40	br s			8.60	m	
19	168.2				168.4			
20	60.3	4.19	t; 9.5		49.3	4.59	m	
20NH		8.47	d; 7.5			8.9	m	
21	26.3	1.86	m		13.9	1.14	d; 6.5	C19, C20
22	18.4	0.92	d; 6.5	C20, C21, C23				
23	18.2	0.86	d; 6.5	C20, C21, C22				

[a] Reference: DMSO-d_6 ^{1}H 2.6 ppm, ^{13}C 39.6 ppm. [b] Water signal interfering. [c] Signals overlapping.

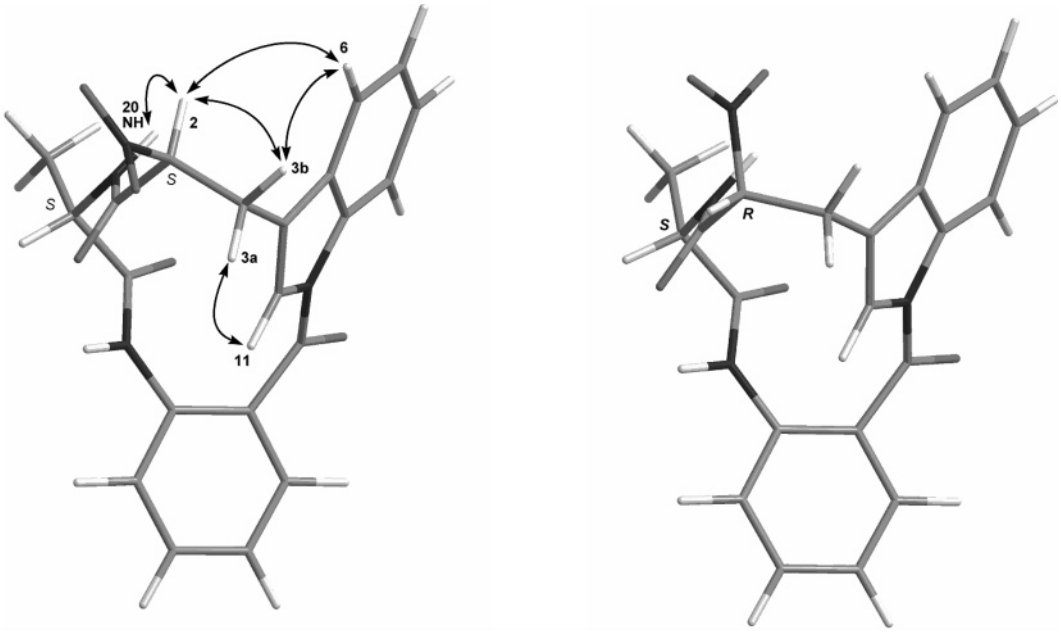

Figure 1. 3D models of the minimal energy conformation of (2*S*,20*S*) and (2*R*,20*S*) psychrophilin C (**2**). The NOE connectivities, shown with arrows, indicate that C-2 most likely has the *S* configuration. Right: 2*S*,20*S* configuration and minimal energy conformation. Left: 2*R*,20*S* configuration and minimal energy conformation.

MHz spectrometer. ^{13}C NMR was recorded on a Varian UNITY 300 MHz spectrometer. Analytical HPLC data were obtained on an Agilent 1100 HPLC-system using Chemstation software and a Phenomenex (Torrance, CA) Luna C_{18} II column (100 × 2 mm, 3 μm) and a Phenomenex SecurityGuard C_{18} precolumn using a linear water−acetonitrile gradient starting with 85% H_2O and 15% acetonitrile (MeCN) and going to 100% MeCN in 20 min, keeping 100% MeCN for 5 min at a flow of 0.4 mL/min at 40 °C. Both solvents contained 50 ppm TFA. Analytical HPLC on the Marfey's derivatives was obtained on a Dionex HPLC system using Chromeleon software version 6.5 and a Phenomenex Jupiter 4u Proteo 90A C_{18} column (250 × 4.6 mm, 4 μm) at a flow of 1 mL/min at 40 °C. HRESIMS analyses were performed using a Micromass TOF LCT mass spectrometer. Data were acquired and processed using the MassLynx program. HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD). The apparatus consisted of three coils, connected in series (total volume, ~850 mL). Solvents for extraction and chromatography were distilled prior to use. The culture medium was Czapek yeast autolysate (CYA). Marfey's reagent [N_α-(2,4-dinitro-5-flourophenyl)-L-ananinamide], (*R*)- and (*S*)-alanine, and (*R*)- and (*S*)-valine were purchased from Sigma.

Fungal Material and Fermentation. The isolate of *Penicillium rivulum* (IBT 24420) was obtained from the IBT Culture Collection at BioCentrum-DTU, Technical University of Denmark. Isolates were cultured on LECA nuts covered by

CYA (4 × 500 mL) at 20 °C for 19 days in the dark. A voucher specimen is located in the collection at the Danish Technical University as IBT 24420.

Extraction and Separations. All the mycelium and agar were extracted twice overnight with EtOAc. After filtration through a Whatman 1PS phase separation filter, the extract was evaporated in vacuo, leaving the crude extract (2 g).

A part of the crude extract was directly separated using a PharmaTech CCC-1000 HSCCC [*n*-heptane−EtOAc−MeOH−H_2O (1:1:1:1), mobile phase: upper phase, tail to head, 850 mL coils, flow 5 mL/min] connected to a Waters pump and a Waters diode array detector. The crude fraction (800 mg) was dissolved in mobile phase (*n*-heptane−EtOAc (1:1)) (30 mL). Twenty-four fractions (50 mL) were collected. Fractions 14 and 15 and fractions 22 and 23 were further purified by HPLC on a preparative Phenomenex Luna 5μ C18 column (250 × 10.00 mm, 5 μm) (flow rate 5 mL/min). Fractions 14 and 15 eluted with MeCN−H_2O (55:45) to afford pure **1** (9.4 mg), and fractions 22 and 23 eluted with MeCN−H_2O (1:1) to afford pure **2** (4.2 mg).

Psychrophilin B (1): white powder from MeCN−H_2O; mp 186−190 °C; $[\alpha]^{25}_D$ +15.2° (*c* 0.033, MeCN); CD λ ext (*c* 0.0033, MeCN) ($\Delta\epsilon$) 222 (−18.3), 229 (−21.1), 239 (−17.4), 276 (5.7), 288 (7.4), 314 (−1.3) nm; UV (MeCN) λ_{max} (log ϵ) 214 (4.27), 244 (4.09), 302 (3.65); IR (KBr) ν_{max} 1366, 1551 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; CIGAR, see Table 1; NOE, see Figure 1; HREISMS obsd $(M + H)^+$ at *m/z* 435.1668, calcd for $C_{23}H_{23}N_4O_5$ 435.1668.

Hydrolysis of Psychrophilin B (1). Psychrophilin B (200 μg) was treated at 155 °C for 60 min with 6 M HCl. After cooling, the sample was freeze-dried and derivatized with Marfey's reagent.[4] The configuration of valine was determined using a H_2O (0.05% TFA)−MeCN gradient (start, 90:10; end, 50:50) over 40 min. Retention times (in min) for the standards were valine, *S*, 29.3, *R*, 33.6. Analysis of the derivative gave a retention time of 29.3 min, establishing an *S* configuration for the valine residue.

Psychrophilin C (2): white powder from MeCN−H_2O; mp 167−171 °C; $[\alpha]^{25}_D$ +16.7° (*c* 0.024, MeCN); CD λ ext (*c* 0.0024, MeCN) ($\Delta\epsilon$) 223 (−17.1), 230 (−18.7), 239 (−12.1), 275 (4.0) 288 (5.2), 315 (−0.3) nm; UV (MeCN) λ_{max} (log ϵ) 217 (4.40), 244 (4.17), 302 (3.71); IR (KBr) ν_{max} 1366, 1555 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; CIGAR, see Table 1; NOE, see Figure 1; HREISMS obsd $(M + Na)^+$ at *m/z* 429.1175, calcd for $C_{21}H_{18}N_4O_5Na$ 429.1175.

Hydrolysis of Psychrophilin C (2). Psychrophilin C (200 μg) was treated at 155 °C for 60 min with 6 M HCl. After cooling, the sample was freeze-dried and derivatized with Marfey's reagent.[4] The configuration of alanine was determined using a gradient of H_2O (0.05% TFA)−MeCN (start, 90:10; end, 50:50) for 40 min. Retention times (in min) for the standards were (*S*)-alanine 22.6, (*R*)-alanine 25.9. Analysis of the derivative gave a retention time of 22.6 min, establishing an *S* configuration for the alanine residue.

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