Psychrophilin A and Cycloaspeptide D, Novel Cyclic Peptides from the Psychrotolerant Fungus *Penicillium ribeum*

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Received August 13, 2003

Two fungal metabolites, psychrophilin A (**1**) and cycloaspeptide D (**2**), together with the known cycloaspeptide A (**3**) were isolated from the psychrotolerant fungus *Penicillium ribeum* using high-speed countercurrent chromatography (HSCCC) and preparative HPLC. The structures were determined from 1D and 2D NMR techniques, HREIMS, tandem mass spectrometry (ESMS/MS), and X-ray crystallography. The amino acid residues of psychrophilin A (**1**) and cycloaspeptide D (**2**) were all found to possess the L configuration by Marfey's method. Psychrophilin A (**1**) is the first natural cyclic peptide containing a nitro group instead of an amino group.

A number of yet undescribed psychrophilic and psychrotolerant filamentous fungi within the genera of *Penicillium* and *Aspergillus* were collected worldwide. Psychrotolerant species grow and sporulate well at 25 °C, but also at lower temperatures, for example 5 °C, whereas psychrophilic species do not grow at 25 °C.[1] The fungi were screened for known and new metabolites using HPLC- and LCMS-screening methods.[2,3] During this endeavor one of the new species, *Penicillium ribeum*,[4] which originates from Wyoming, was found to contain compounds identical or related to the cycloaspeptides, previously only isolated from an unidentified *Aspergillus* sp.[5]

An EtOAc extract of *Penicillium ribeum* (IBT 16537) was separated by high-speed countercurrent chromatography (HSCCC),[6] and the constituents were purified by preparative HPLC. Two of the resulting compounds were identified as new cyclic peptides and named psychrophilin A (**1**) and cycloaspeptide D (**2**) (Figure 1). The third compound was identified as cycloaspeptide A (**3**).[5]

Psychrophilin A (**1**) exhibited a molecular ion in HREIMS corresponding to the molecular formula $C_{23}H_{20}N_4O_5$. The ^{13}C NMR and ^1H NMR (Table 1) spectra revealed resonances consistent with three amide carbonyls (δ 167.0, 166.3, and 163.9), two α-methine carbons (δ 83.7 and 59.2), and ortho-substituted benzene and indole ring systems, suggesting a peptide with an anthranilic acid (ABA) and a tryptophan. The amino acids were identified by 2D NMR techniques. One independent spin system of the type X−CH−CH_2−CH_2−CH_2−X′ was defined as originating from proline, and another independent spin system of the type X−CH−CH_2−X′ was attributed to tryptophan using COSY, ROESY, HMQC, and HMBC correlations.

The surprisingly low-field proton chemical shift at δ 6.06 was assigned to a transformed tryptophan residue where the α-amino group was oxidized to a nitro group. The presence of a nitro group was substantiated by the N−O stretching vibration at 1554 and 1360 cm^{-1}. This result in combination with the fact that the peptide must possess a cyclic structure demands three peptide bonds connecting the Trp derivative with the ABA unit to encompass the indole nitrogen.The amino acid sequence of psychrophilin A (**1**) was established as *cyclo*-(nitrotryptophan-prolyl-*o*-aminobenzoyl) [cyclo-(nitro-Trp-Pro-ABA)] by the NOE and HMBC correlations (Figure 2). The relative configuration was established by X-ray crystallography. The molecular structure of psychrophilin A as observed in the crystal is shown in Figure 3[7] and reveals that the two chiral centers have the same configurations, *S*,*S* or *R*,*R*.

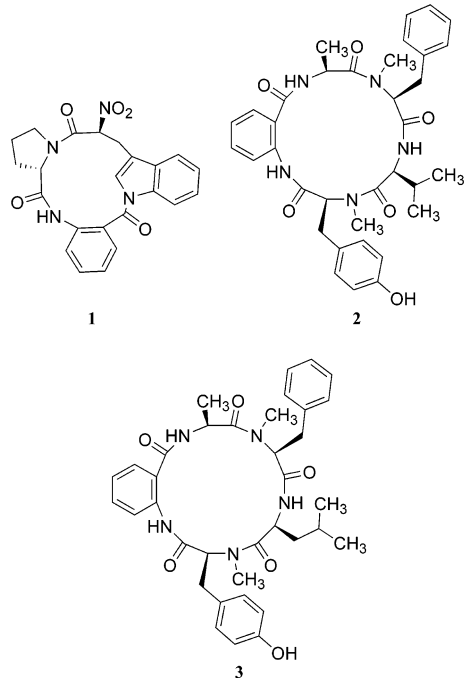

Figure 1. Psychrophilin A (**1**), cycloaspeptide D (**2**), and cycloaspeptide A (**3**) isolated from *Penicillium ribeum*.

The absolute stereochemistry of psychrophilin A (**1**) (*S*,*S*) was determined from a Marfey[8] analysis of proline, formed by hydrolysis of psychrophilin A (**1**). Comparison of HPLC retention times of the derivative of proline with L- and D-proline standards unambiguously proved proline to have the L-configuration.

Cycloaspeptide D (**2**) exhibited a molecular ion in HREIMS corresponding to the molecular formula $C_{35}H_{41}N_5O_6$.

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10.1021/np0303714 CCC: $27.50
Published on Web 03/26/2004

Table 1. NMR Data for Psychrophilin A (**1**) (400 MHz (^{1}H), 100.6 MHz (^{13}C), in DMSO-d_6)

residue	position	δ_C	δ_H
proline	Cα	59.2	4.65 (1H, dd, J = 7.9/3.4 Hz)
	Cβ	25.5	1.85 (1H, m), 1.74 (1H, m)
	Cχ	24.3	1.54 (1H, m), 1.33 (1H, m)
	Cδ	47.1	2.01 (1H, m), 3.30 (1H, dd, 17.5/7.2 Hz)[a]
	CO	167.0	
tryptophan	NO_2		
	Cα	83.7	6.06 (1H, dd, J = 11.3/5.3 Hz)
	Cβ	26.0	3.65 (1H, dd, J = 12.6/5.1 Hz), 3.40 (1H, t, J = 11.9 Hz)[a]
	N-1		
	C-2	126.5	6.89 (1H, s)
	C-3	113.0	
	C-3a	129.5	
	C-4	118.9	7.84 (1H, d, J = 7.5 Hz)
	C-5	123.8	7.35 (1H, t, J = 7.4 Hz)
	C-6	125.2	7.39 (1H, m)[b]
	C-7	116.4	8.46 (1H, d, J = 8.2 Hz)
	C-7a	134.9	
	CO	163.9	
o-aminobenzoic acid	NH		9.59 (1H, s)
	C-6	131.9	7.72 (1H, d, J = 7.7 Hz)
	C-5	125.5	7.39 (1H, m)[b]
	C-4	132.4	7.66 (1H, s)[b]
	C-3	122.9	7.65 (1H, s)[b]
	CO	166.3	
	C-2	133.8	
	C-1	126.6	

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

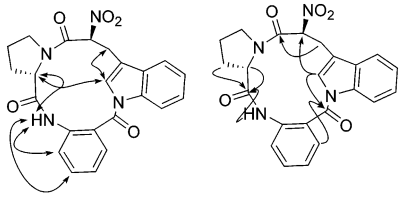

Figure 2. Selected connectivities in psychrophilin A (**1**). (Left) ROESY. (Right) HMBC.

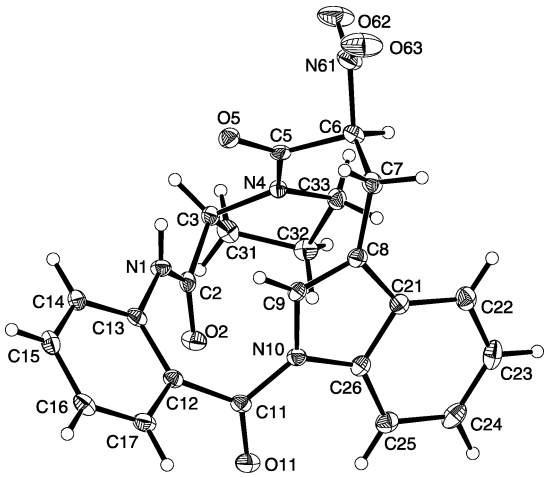

Figure 3. Molecular structure (ORTEPII[6]) of psychrophilin A with atom-labeling scheme for non-hydrogen atoms. Displacement ellipsoids of the non-hydrogen atoms are shown at the 50% probability level. Hydrogen atoms are represented by spheres of arbitrary size.

The ^{13}C NMR and ^{1}H NMR spectra (Table 2) displayed signals from five amide carbonyl carbons (δ 173.1, 169.0, 168.9, 168.5, and 168.4), four α-methine carbons (δ 68.5, 62.7, 55.2, and 43.7), three amide protons (δ 11.89, 8.80, and 8.15), two *N*-methyl groups (δ 2.76 and 2.63), one phenyl, one para-substituted phenyl, and one ortho-substituted benzene, suggesting a pentapeptide with phenylalanine, tyrosine, and anthranilic acid. The spin system X−CH−CH(CH$_3$)$_2$ and X−CH−CH$_3$ was identified, suggesting the existence of valine and alanine. The remaining two independent spin systems of the type X−CH$_2$−CH−X′ were attributed to phenylalanine and tyrosine by ROESY. Phe and Tyr were found to be *N*-methylated by HMBC between *N*-methyl protons and the nearest carbonyl carbon (alanine and valine) and the nearest α-carbon (phenylalanine and tyrosine).

Table 2. NMR Data for Cycloaspeptide D (**2**) 400 MHz (^{1}H), 100.6 MHz (^{13}C), in DMSO-d_6

residue	position	δ_C	δ_H
alanine	NH		8.80 (1H, d, J = 5.5 Hz)
	Cα	43.7	4.43 (1H, t, J = 6.2 Hz)
	Cβ	15.8	0.41 (3H, d, J = 6.6 Hz)
	CO	173.1	
phenylalanine	N-Me	30.8	2.76 (3H, s)
	Cα	62.7	5.37 (1H, dd, J = 11.5/3.1 Hz)
	Cβ	34.1	3.39 (1H, dd, J = 14.0/2.8 Hz), 2.87 (1H, dd, J = 14.0/12.0 Hz)
	C1	138.3	
	C2−5	129.0	7.28 (4H, m)
	C-6	126.5	7.22 (1H, m)
	CO	168.9	
valine	NH		8.15 (1H, d, J = 9.0 Hz)
	Cα	55.2	4.20 (1H, t, J = 8.8 Hz)
	Cβ	29.8	2.12 (1H, m)
	Cγ	18.5	0.96 (3H, d, J = 6.4 Hz)
	Cγ	20.6	0.86 (3H, d, J = 7.0 Hz)
	CO	169.0	
tyrosine	N-Me	38.6	2.63 (3H, s)
	Cα	68.5	4.12 (1H, dd, J = 10.9/4.1 Hz)
	Cβ	31.7	3.25 (1H, dd, J = 13.9/4.0 Hz), 3.07 (1H, dd, J = 13.9/11.0 Hz)
	C1	130.1	
	C2, C6	129.9	7.00 (2H, d, J = 8.4 Hz)
	C3, C5	115.2	6.71 (2H, d, J = 8.4 Hz)
	C4	155.8	
	CO	168.5	
	OH		9.26 (1H, br s)
o-aminobenzoic acid	NH		11.89 (1H, s)
	C6	118.8	8.75 (1H, dd, J = 8.4/0.9 Hz)
	C3	128.6	7.95 (1H, dd, J = 8.1/1.3 Hz)
	C5	132.9	7.50 (1H, ddd, J = 8.4/7.1/1.2 Hz)
	C4	121.6	7.05 (1H, ddd, J = 8.2/7.1/1.1 Hz)
	CO	168.4	
	C2	140.6	
	C1	116.8	

The ROESY experiment indicated the Ala and the Phe residues to be linked because of the presence of the correlation between both α-protons and the aromatic protons and one methyl group (Figure 4). This result was confirmed by HMBC, which also served to place one of the *N*-Me groups on Phe. The Ala and Phe carbonyl groups showed long-range coupling to the same *N*-Me group. The remaining connectivities were interpreted analogously, giving rise to the structure shown.

The amino acid residues accounted for 17 out of the 18 degrees of unsaturation, thus demanding **2** to assume the structure of a cyclic pentapeptide. The amino acid sequence of cycloaspeptide D (**2**) was deduced from a combination of 2D NMR and electrospray tandem mass spectrometry techniques (ESMS/MS) data. A ROESY experiment indicated the Ala and the Phe residues to be linked because of the presence of the correlation between both α-protons and the aromatic protons and one methyl group (Figure 4). This result was confirmed by HMBC, which also served to place one of the *N*-Me groups on Phe. The Ala and Phe carbonyl groups showed long-range coupling to the same *N*-Me group. The remaining connectivities were interpreted analogously, giving rise to the structure shown.

Now the complete sequence of cycloaspeptide D (**2**) could be deduced on the basis of the results of ESMS/MS experiments, where the fragmentation patterns confirmed the close relationship between the known cycloaspeptide A (**3**) and the new cycloaspeptide D (**2**). Table 3 lists the daughter ions of the singly charged ion at *m*/*z* 628 corre-

Table 3. Cycloaspeptide D (**2**) and A (**3**) Showed Comparable Fragmentation Patterns in the Spectra of the Collision-Activated Pseudo-Molecular Ion ($[M + H]^+$) in ESMS/MS[a]

cycloaspeptide D (**2**)		cycloaspeptide A (**3**)	
m/z	pseudo-molecular ion − amino acids	*m/z*	pseudo-molecular ion − amino acids
628	$[M+H]^+$	642	$[M+H]^+$
529	$[M+H]^+$ − Val	529	$[M+H]^+$ − Leu
451	$[M+H]^+$ − NMeTyr	465	$[M+H]^+$ − NMeTyr
438	$[M+H]^+$ − ABA − Ala	452	$[M+H]^+$ − ABA − Ala
368	$[M+H]^+$ − NMePhe − Val	368	$[M+H]^+$ − NMePhe − Leu
352	$[M+H]^+$ − Val − NMeTyr	352	$[M+H]^+$ − Leu − NMeTyr
332	$[M+H]^+$ − NMeTyr − ABA	346	$[M+H]^+$ − NMeTyr − ABA
297	$[M+H]^+$ − Ala − NMePhe -Val	297	$[M+H]^+$ − Ala − NMePhe − Leu
261	$[M+H]^+$ − NMeTyr − ABA − Ala	275	$[M+H]^+$ − NMeTyr − ABA − Ala
233	$[M+H]^+$ − Val − NMeTyr − ABA	233	$[M+H]^+$ − Leu − NMeTyr − ABA
162	$[M+H]^+$ − Val − NMeTyr − ABA − Ala	162	$[M+H]^+$ − Leu − NMeTyr − ABA − Ala

[a] The only difference was due to the exchange between Val and Leu. ABA = *o*-aminobenzoic acid.

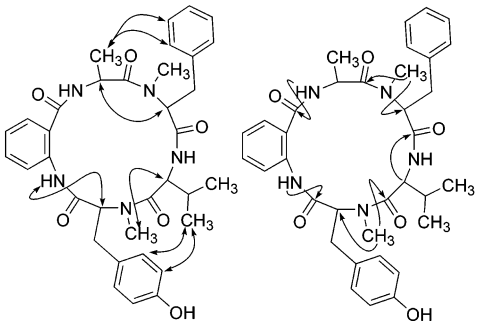

Figure 4. Selected connectivities in cycloaspeptide D (**2**). (Left) ROESY. (Right) HMBC.

sponding to the pseudo-molecular ion of **2** and *m/z* 642 derived from cycloaspeptide A (**3**). The fragmentation process generated a complex pattern of fragment ions mainly belonging to the b series,[9] although some ions of the a series were also observed.

To determine the absolute stereochemistry, cycloaspeptide D (**2**) was hydrolyzed and the resulting amino acids were derivatized according to Marfey.[8] Comparison of the HPLC retention time of the derivatives with standards served to clarify the absolute configuration of the derivatives. Accordingly, the structure of cycloaspeptide D (**2**) was determined as *cyclo*-(L-alanyl-L-*N*-methylphenylalanyl-L-valyl-L-*N*-methyltyrosyl-*o*-aminobenzoyl).

Comparison with literature data revealed **3** to be identical to cycloaspeptide A.[5] Owing to the anthranilic acid component and the *N*-methylation pattern present in psychrophilin A and the cycloaspeptides, we submit that these peptides originate from a nonribosomal source, viz., are products of peptide synthetase enzymes.[10] This deduction opens the possibility of manipulating the synthesis of variations of these peptides by introducing different amino acids in the cyclic structure. This potential biocombinatorial system is under investigation.

In this paper, we have reported the first naturally occurring cyclic peptide with a NO_2 group. Aliphatic nitro groups are rare as secondary metabolites in nature, and a cyclic peptide containing an aliphatic nitro group incorporated the same way as in psychrophilin A has never before been reported. We submit that the nitro group is formed by enzymatic oxidation.

Experimental Section

General Experimental Procedures. The circular dichroism (CD) spectrum was measured on a modified JASCO 710 instrument. The UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. NMR spectra were recorded in DMSO-d_6 on a Varian 400 FT-NMR spectrometer operated at 400.0 and 100.6 MHz for ^1H and ^{13}C NMR spectra, respectively. The HPLC data were obtained on an Agilent 1100 HPLC system using Chemstation software and a Hewlett-Packard Hypersil BDS-C18, 4 μm, 4.0 × 100 mm column; flow 1 mL/min. Electrospray tandem mass spectrometric (ESMS/MS) analyses were performed using a Q-ToF quadropole mass spectrometer (Micromass, Manchester, UK). Aliquots of the cycloaspeptides were injected in the ion source at a flow rate of 5 μL/min [75% MeOH−25% H_2O (0.5% HCOOH and 0.1% HCl)]. Scanning was performed from *m/z* 100 to *m/z* 700 at 1 s/scan, using a cone voltage of 40 V. Fragmentation experiments were carried out using nitrogen as collision gas and a collision energy of 40 eV. Data were acquired and processed using the MassLynx program (Micromass, Manchester, UK). HREIMS mass spectra were recorded on a JEOL JMS_MX/HX 110A.

Marfey's reagent [N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide], D- and L-alanine, D- and l-valine, and D- and L-proline were purchased from Sigma. D- and L-*N*-methylphenylalanine and D- and L-*N*-methyltyrosine were purchased from Bachem.

Collection, Isolation, and Fermentation. *Penicillium ribeum* (IBT 16537) was collected from a soil under a *Ribes* sp. at the summit of Eagle Rock, Medicine Bow National Forest in Wyoming by Jens C. Frisvad, September 11, 1994. The fungus produced a series of compounds with UV spectra resembling that of cycloaspeptides on Czapek yeast autolysate agar (CYA)[11] as determined by HPLC.[2] The fungus was cultivated as five-point mass inoculation, 250 Petri dishes containing CYA at 25 °C for 10 days in the dark. A voucher specimen is located in the IBT Collection at BioCentrum at the Danish Technical University as IBT 16537.

Extraction and Isolation. The contents of 250 Petri dishes were transferred to four large glass flasks and were extracted at still conditions for 16 h at room temperature with 2.5 L of EtOAc to give 2.4 g of dried extract after evaporation of EtOAc. The crude extract was directly separated using a PharmaTech CCC-1000 HSCCC [*n*-heptane−EtOAc−MeOH−water (1:1:1:1), mobile phase: upper phase, (T) → H, 325 mL coils, flow 3 mL/min] connected to Water pumps and diode array detector. Crude fractions (200−250 mg) were dissolved in a 30 mL mobile phase (*n*-heptane−EtOAc (1:1)). The HSCCC separation was repeated several times. Four fractions were collected using UV-guided fractionation. The second (**1**), third (**2**), and fourth (**3**) fractions were further purified by HPLC on a preparative (7.8 × 300 mm) Waters Symmetry-C18 column (flow rate 4 mL/min) eluted with MeCN−H_2O (60:40) mixtures to afford pure **1** (48 mg), **2** (19 mg), and **3** (7 mg).

Psychrophilin A (1): colorless solid; mp 164−167 °C; $[\alpha]^{25}_D$ 43.7° (MeCN); CD λ_{ext} (*c* 0.003 MeCN) ($\Delta\epsilon$) 217.5 (−287.9), 224 (−248.4), 230.5 (278.0), 275.5sh (77.6), 288.5 (85.8), 319.5 (−15.3) nm; UV (MeCN) λ_{max} (log ϵ) 217 (4.37), 244 (4.15), 304 (3.74) nm; IR (KBr) ν_{max} 1554, 1360 cm^{-1}; NMR data, see Table 1; HREIMS *m/z* 432.1431 (calcd 432.1434 for $C_{23}H_{20}N_4O_5$).

Hydrolysis of Psychrophilin A (1). The compound (200 μg) was treated at 165 °C for 25 min with 6 N HCl. After cooling, the sample was freeze-dried and derivatized with

Marfey's reagent.[8] The configuration of Pro was determined by using a gradient of H_2O (0.1% TFA)/MeCN (0.1% TFA) (start, 90:10; end, 50:50) for 40 min. Retention times (in min) for the standards were Pro, L, 15.7, D, 17.2. The analysis gave a retention time (in min) of 15.7, establishing the L-configuration for the Pro residue.

X-ray Crystallographic Analysis of Psychrophilin A (1). Suitable colorless single crystals were obtained from a solution of the compound in MeOH and selected for data collection. Crystal dimensions: 0.17 × 0.19 × 0.22 mm. Crystal data: $C_{23}H_{20}N_4O_5$, M_r = 432.43, orthorhombic, space group $P2_12_12_1$ (No. 19), a = 7.6077(5) Å, b = 8.7862(9) Å, c = 29.906(3) Å, V = 1999.0(3) Å^3, Z = 4, D_c = 1.437 Mg/m^3, $F(000)$ = 904, μ(Mo Kα) = 0.104 mm^{-1}, T = 122(1) K. Diffraction data were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo Kα radiation (λ = 0.71073Å). Data collections and cell refinements were performed using COLLECT[12] and DIRAX.[13] The reflections were measured in the range $-9 \le h \le 9$, $-10 \le k \le 10$, $-35 \le l \le 35$ ($2.42° < \theta < 24.99°$). Data reductions were performed using EvalCCD.[14] A total of 31 603 reflections were averaged[15,16] according to the point group symmetry 222, resulting in 3507 unique reflections (R_{int} = 0.044 on F^2).

The structure was solved by direct methods, SHELXS97,[17,18] and structure refinements were performed with SHELXL97.[19] Full matrix least-squares refinement on F^2 was performed, minimizing $\sum w(F_o^2 - F_c^2)^2$, with anisotropic displacement parameters for the non-hydrogen atoms. The positions of all hydrogen atoms were located on intermediate difference electron density maps. The positions of hydrogen atoms on the aromatic rings and the methylene groups have been refined riding on the parent atoms, while the positions of the rest of the hydrogen atoms have been refined independently. Isotropic displacement parameters for all hydrogen atoms have been fixed (1.2U_{eq} of the parent atoms). The refinement (301 parameters, 3507 reflections) with the molecule having the *S*,*S* absolute configurations as shown in Figure 3 converged at R_F = 0.0305, wR_F^2 = 0.0661 for 3183 reflections with [$I > 2\sigma(I)$]; $w = 1/[\sigma^2(F_o^2) + (0.0314P)^2 + 0.5127P]$, where $P = (F_o^2 + 2F_c^2)/3$; S = 1.113. In the final difference Fourier map maximum and minimum electron densities were 0.155 and −0.211 e·Å^{-3}, respectively. The absolute configuration could not be determined reliably, due to low anomalous scattering of the present atom types. The relative configuration can be assigned to be either *S*,*S* or *R*,*R*. Atomic scattering factors for neutral atoms were used as incorporated in SHELXL97.[19,20]

Cycloaspeptide D (2): colorless solid; mp 186−189 °C; $[\alpha]^{25}_D$ −41.0 (MeCN); CD λ_{ext} (*c* 0.078, MeCN) ($\Delta\epsilon$) 202.5 (19.3), 223 (−40.9), 256 (−10.5), 262.5sh (−8.9), 314 (−0.9) nm; UV (MeCN) λ_{max} (log ϵ) 216 (0.112), 256 (0.293), 285 (2.47), 307 (0.112) nm; NMR data, see Table 2; HREIMS showed a signal at *m*/*z* 627.3060 (calcd 627.3057 for $C_{35}H_{41}N_5O_6$).

Hydrolysis of Cycloaspeptide D (2). The compound (200 μg) was treated at 155 °C for 1 h with 6 N HCl. After cooling, the sample was freeze-dried and derivatized with Marfey's reagent.[8] The configurations of Ala, Val, *N*-Me-Phe, and *N*-Me-Tyr were determined by using a gradient of H_2O (0.1% TFA)/MeCN (0.1% TFA) (start, 90:10; end, 50:50) for 40 min. Retention times (in min) for the standards were Ala, L, 12.2, D, 15.8; Val, L, 18.6, D, 23.4; *N*-Me-Phe, L, 24.2, D, 24.5; *N*-Me-Tyr, l, 29.9, D, 30.4. The analysis gave retention times (in min) of 12.2, 18.5, 24.2, and 30.0, establishing the L-configuration for all amino acid residues.

Acknowledgment. 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 H. Jacobsen for support with the analytical HPLC, the LC MS, and Q-ToF analysis. J. C. Frisvad carried out the taxonomical studies of the fungus. The technical assistance of F. Hansen with the X-ray data collection is gratefully acknowledged.

Supporting Information Available: Crystal and structure refinement data, final atomic coordinates, equivalent isotropic displacement parameters for non-hydrogen atoms, bond lengths and angles, anisotropic displacement parameters for non-hydrogen atoms, final atomic coordinates with fixed isotropic displacement parameters for hydrogen atoms, torsion angles, intramolecular hydrogen bonds, and crystal packing diagram of psychrophilin A. This material is available free of charge via the Internet at http://pubs.acs.org.[21]

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(21) Crystallographic data for the structure of psychrophilin A reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (code CCDC214288). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK; fax: +44 (0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk.

NP0303714