Characterization of Pseudacyclins A−E, a Suite of Cyclic Peptides Produced by *Pseudallescheria boydii*

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Pseudallescheria boydii sensu lato is an emerging fungal pathogen causing fatal infections in both immunocompromised and immunocompetent hosts. In this work, two *P. boydii* isolates (human and animal origin) have been identified as being producers of cyclic peptides. Five putative nonribosomal peptides with a unique structure, which have been named pseudacyclins, were characterized by nuclear magnetic resonance spectroscopy and mass spectrometry. The most abundant representative of the pseudacyclins was quantified also on fungal spores. The presence of these peptides on inhaled fungal spores creates the possibility for exploitation of pseudacyclins as early indicators of fungal infections caused by *Pseudallescheria* species.

Infections caused by the *Pseudallescheria boydii sensu lato* fungal complex have been classified as opportunistic systemic mycoses causing fatal invasive infections in immunocompromised and immunocompetent hosts.[1−3] The fungal strains involved in the complex exert very high levels of antifungal resistance,[4] with *Scedosporium aurantiacum* being the most resistant species.[5] *P. boydii* has now become the second most frequent filamentous mold recovered from cases involving particular patient groups, e.g., colonizing the respiratory tract of cystic fibrosis patients or causing delayed brain infection in the near drowning syndrome.[6,7]

P. boydii sensu lato is a complex of fungal species currently involving *P. angusta, P. boydii sensu stricto, P. desertorum, P. elipsoidea, P. fusoidea, P. minutispora, Scedosporium apiospermum, S. aurantiacum*, and *S. dehoogii.*[8] *P. boydii* and *S. apiospermum*, traditionally considered as the teleomorph and anamorph of the same species, in fact belong to two different species. The high mortality rate related to *S. prolificans* infection is alarming, exceeding 87% in patients with disseminated disease.[4] *S. prolificans* was reported to be more virulent than *S. apiospermum*. In the animal studies *S. aurantiacum* and *S. dehoogii* killed 70−80% of the immunocompetent mice, whereas *P. boydii sensu stricto*, *P. minutispora*, and *S. apiospermum* killed only 0−20% of the animals.[9]

Although *P. boydii* was first described in human disease in India as one of the agents of mycetoma more than 150 years ago and the first infection caused by *Scedosporium* was reported in 1889, a reliable commercially available tool for early diagnosis of infections caused by *P. boydii* complex is still missing. Current experimental tools are based on either *P. boydii* genotyping[10] or detection of saccharidic antigens representing the fungal cell-wall components.[11] Certain skepticism arising from quite low sensitivities of some of these techniques means that cultivation and microscopy of biopsies still represent the gold standard.[12]

Long-standing and/or unreliable diagnostics currently stimulate the analytical community to develop alternative tools that can be used in early stage diagnosing and characterization of infections caused by multiple species acting cooperatively. In genotyping methods nucleotide substitutions do not necessarily result in alteration in the amino acid composition, and subsequent changes in the electrophoretic profile may not be visualized.[10] On the contrary, going down from a gene to a protein/peptide level gives inherently more precise information.[13] For peptide/protein analysis, mass spectrometry is being used as the exclusive tool, which in selected cases makes possible not only the determination of the fungal genus but also distinctions among the different strains.[14] The emerging protocols are mostly based on proteomics or peptidomics approaches.[15] It has to be emphasized that all of the reported successful attempts to date have been made only under *in vitro* conditions. However, the sensitivity of modern molecular tools is enormous: just 10 fungal conidia are necessary for detection of a particular strain even in a fungal complex.[16]

Not many secreted organic substances have been reported specifically for the *P. boydii* complex.[17] A 33 kDa extracellular serine protease/peptidase from *S. apiospermum* was described as degrading human fibrinogen, suggesting a role as a mediator of tissue injury and inflammation.[18] Silva et al. have reported two extracellular metallopeptidases of 28 and 35 kDa that are released from mycelia of *P. boydii.*[19] These peptidases were found to break down portions of fibronectin and laminin.[20]

Oligosaccharidic structures can potentially be used in serologic diagnostics of *P. boydii.*[21,22] Fungal cerebrosides represent another interesting glyco-related subgroup of antigens.[23] A recent paper reports on the preparation of two monoclonal antibodies that selectively bind to a major antigen with a molecular mass of approximately 120 kDa secreted by *P. boydii.*[11] Promising specificities achieved *in vitro* to *Pseudallescheria*, *Graphium*, and *Petriell*a species and *S. apiospermum* remain to be evaluated with clinical samples.

Fungal melanin structures, derived from the precursor molecule 1,8-dihydroxynapthalene through the polyketide biosynthetic pathway, may serve as scavengers for oxygen and nitrogen reactive species produced by phagocytic cells during the oxidative burst and thus contribute to fungal pathogenesis.[24]

Metabonomics represents another modern approach for low molecular mass fungal biomarkers. All of these small organic molecules reported as of July 2009 to be produced by the *P. boydii*

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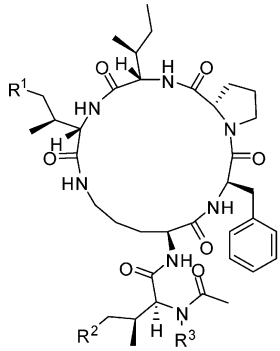
Table 1. Non-saccharidic Small-Molecule Metabolites Described in *Pseudallescheria/Scedosporium* Strains As of July 2009

trivial name	$[M + H]^+$	formula	species	ref
pseurotin A	127.0502	$C_5H_7N_2O_2$	*P. boydii* IFM 4642	27
AS-183	311.2581	$C_{19}H_{35}O_3$	*S.* sp. SPC-15549	28
gliotoxin	327.0468	$C_{13}H_{15}N_2O_4S_2$	*P.* sp. MFB165	26
tyroscherin	334.2741	$C_{21}H_{36}NO_2$	*P.* sp.	29
dehydroxybisdethiobis(methylthio)gliotoxin	341.0988	$C_{15}H_{21}N_2O_3S_2$	*P.* sp. MFB165	26
YM-193221	346.2741	$C_{22}H_{36}NO_2$	*P. ellipsoidea* CBS128.78	30
bisdethiobis(methylthio)gliotoxin	357.0937	$C_{15}H_{21}N_2O_4S_2$	*P.* sp. MFB165	26
dimerumic acid[a]	485.2606	$C_{22}H_{37}N_4O_8$	*S. apiospermum*,e.g., IHEM 14263	31
pseudacyclin E, [Val^5,Val^6]PcA, **5**[b]	712.4392	$C_{37}H_{58}N_7O_7$	*P. boydii* CBS 119458	this work
pseudacyclin B, [Val^5]PcA, **2**	726.4549	$C_{38}H_{60}N_7O_7$	*P. boydii* CBS 119458	this work
pseudacyclin D, [Val^6]PcA, **4**	726.4549	$C_{38}H_{60}N_7O_7$	*P. boydii* CBS 119458	this work
pseudacyclin A, **1**	740.4705	$C_{39}H_{62}N_7O_7$	*P. boydii* CBS 119458, CBS 116779	this work
2-*N*-methylcoprogen B[a]	741.4029	$C_{34}H_{57}N_6O_{12}$	*S. aurantiacum* *S. apiospermum*	31
pseudacyclin C, [Me-acetyl-Ile^6]PcA, **3**	754.4858	$C_{40}H_{64}N_7O_7$	*P. boydii* CBS 119458	this work

[a] In the literature the siderophores dimerumic acid and 2-*N*-methylcoprogen B were reported as $[M + Fe - 2H]^+$ species (*m/z* 538.1721 and 794.3144, respectively). [b] PcA stands for pseudacyclin A.

complex are summarized in Table 1. Some of these are thought to be important virulence factors, e.g., the gliotoxin family.[25,26] In this work we have focused on characterization of the five cyclic peptides with unique structures and produced by two *Pseudallescheria* strains. The structure of the most abundant cyclopeptide belonging to this family has been named pseudacyclin A (**1**).



	R^1	R^2	R^3
Pseudacyclin A (**1**)	CH_3	CH_3	H
Pseudacyclin B (**2**)	H	CH_3	H
Pseudacyclin C (**3**)	CH_3	CH_3	CH_3
Pseudacyclin D (**4**)	CH_3	H	H
Pseudacyclin E (**5**)	H	H	H

Results and Discussion

Two *P. boydii* isolates, one of human and the other of animal origin, have been identified as cyclic peptide producers. Upon receipt from Centraalbureau voor Schimmelcultures (CBS), both *P. boydii* strains were checked for their authenticity by rDNA sequencing. A high similarity in the ITS1-5.8S-ITS2 region between CBS 119458 and CBS 116779 was observed (data not shown). *P. boydii* CBS 119458 strain was also checked by surface culture and microscopic tools (see the Supporting Information).

Fungal strains CBS 119458 and CBS 116779 were shown to produce pseudacyclin A (**1**), a new cyclic hexapeptide containing three isoleucine residues (one of which is *N*-acetylated) and one residue each of phenylalanine, ornithine, and proline. The molecule presents a unique structure of a type previously not reported. The structure was determined by 2D NMR spectroscopy as 2-acetylamino-3-methylpentanoic acid (5-benzyl-14,17-di-*sec*-butyl-4,7,13,16,19-pentaoxo-eicosahydro-3a,6,12,15,18-pentaaza-cyclopentacyclooctadecen-8-yl)amide. NMR analyses based on a combination of 1D and 2D NMR experiments determined six subunits constituting compound **1**: ornithine (Orn^1), phenylalanine (Phe^2), proline (Pro^3), and three isoleucines (Ile^4, Ile^5, Ile^6). *N*-Acetylation of Ile^6 was confirmed by HMBC correlations of the methyl signal at 1.97 ppm and an amide NH at 7.03 ppm to the carbonyl carbon at 172.2 ppm (Table 2). The sequence determination of **1** was accomplished by HMBC using correlations between the secondary amide proton and the corresponding carbonyl carbon resonances. It gained the contiguous sequence of residues −Pro^3-Ile^4-Ile^5-*N*δ-Orn^1(N^1-*N*-Ac-Ile^6)-Phe^2−. The cyclic structure was also confirmed by negative carboxypeptidase Y treatment as well as resistance to the esterification by ethanol (data not shown). Enantioselective analysis of **1** has been performed according to protocol[32] and has shown the L configuration for all amino acids except for D-Phe-2.

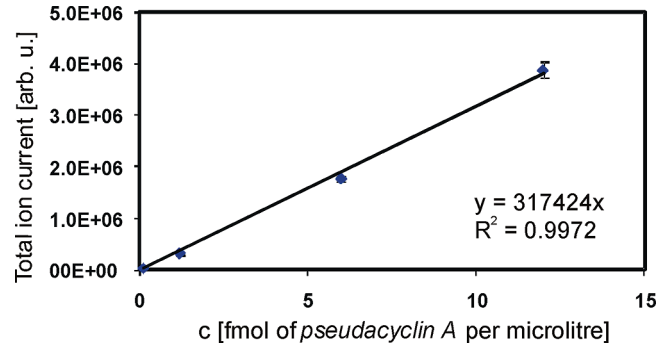


Figure 1. Standard calibration curve relating a total-ion-current mass-detector signal to the concentration of pseudacyclin A in femtomoles per microliter (LCQ-DECA ion trap, SRM mode, parent ion *m/z* 740.5 selected with a 5 Th window, product ion range scanned between 582 and 588 Th).

A higher production of pseudacyclin A was observed with the second strain CBS 119458. In this particular case, the cyclopeptide was also detected on the fungal spores (for spore treatment protocol see ref 15). Semiquantitative measurements by mass spectrometry indicated that there was 5×10^{-20} mol of pseudacyclin A on one CBS 119458 spore, which corresponds roughly to 30 000 molecules per spore (Figure 1). Mass spectrometry works routinely in the detection range of interest. It should be noted, however, that the reported selected reaction monitoring (SRM) measurement was carried out with peptide standards. The detection limit during a whole blood or a tissue measurement could be hampered by chemical noise and by marker losses caused by extraction procedures. Its actual presence on both fungal spores and growing mycelium could create the possibility for exploiting these unique molecules as early indicators of fungal infections caused by *Pseudallescheria* sp. Pseudacyclins can in principle be detected as either circulating free peptides or protein-bound molecules. In terms of specificity, cyclic peptides might represent a viable alternative to current experimental diagnostic tools based on less specific polysaccharidic antigens. The actual role of cyclic peptides in *P.*

Table 2. NMR Data of Pseudacyclin A (**1**) (399.87 MHz for ^{1}H and 100.55 MHz for ^{13}C, CD_3CN, 303 K).

residue	atom	δ_C[a]	δ_H (*J* in Hz)	HMBC (H to C correlations)
Orn-1	CO	172.4, C		
	NH		7.62, d (8.7)	6-CO
	α	53.7, CH	4.23, ddd (3.3, 8.7, 12.1)	1-CO, 1*β*
	β	27.5, CH_2	1.89[a], m	
			1.46[a], m	1α, 1*γ*, 1*δ*
	γ	25.2, CH_2	1.57[a], m	
			1.26[a], m	
	δ	38.6, CH_2	3.47, m	
			2.77, m	
	δ-NH		7.23[b], m	5-CO
Phe-2	CO	171.8, C		
	NH		7.35, d (7.8)	1-CO
	α	53.6, CH	4.74, ddd (6.6, 7.8, 8.7)	1-CO, 2-CO, 2-*ipso*, 2*β*
	β	38.4, CH_2	2.97, dd (8.7, 13.1)	2-CO, 2-*ipso*, 2-*ortho*, 2α
			2.88, dd (6.6, 13.1)	2-CO, 2-*ipso*, 2-*ortho*, 2α
	ipso	137.9, C		
	ortho	130.2[c], CH	7.22[a], m	2*β*
	meta	129.2[c], CH	7.26[a], m	2-*ipso*, 2-*meta*
	para	127.6, CH	7.23[a], m	
Pro-3	CO	171.7, C		
	α	61.5, CH	4.30, dd (2.8, 8.4)	3-CO, 3*β*, 3*γ*, 3*δ*
	β	30.1, CH_2	1.95[a], m	3-CO
			1.85[a], m	3-CO
	γ	24.8, CH_2	1.69[a], m	
	δ	47.4, CH_2	3.62, ddd (3.7, 7.7, 9.8)	
			2.86, ddd (7.3, 9.0, 9.8)	3*β*, 3*γ*
Ile-4	CO	172.9, C		
	NH		6.55, d (8.9)	3-CO, 4-CO
	α	56.7, CH	4.46, dd (3.5, 8.9)	3-CO, 4-CO, 4*β*, 4*γ*
	β	40.1, CH	1.90, m	
	γ	24.1, CH_2	1.26, m	
			0.94[a], m	
	δ	11.9, CH_3	0.81, t (7.3)	4*β*, 4*γ*
	β-CH_3	16.2, CH_3	0.83, d (6.7)	4α, 4*β*, 4*γ*
Ile-5	CO	171.9, C		
	NH		6.75, d (6.2)	4-CO
	α	61.0, CH	3.86, dd (6.2, 9.0)	5-CO, 5*β*, 5*γ*, 5*β*-CH_3
	β	35.9, CH	1.73[a], m	
	γ	26.0, CH_2	1.60[a], m	
			1.18[a], m	5*β*, 5*δ*, 5*β*-CH_3
	δ	10.8, CH_3	0.87, t (7.3)	5*β*, 5*γ*
	β-CH_3	15.5, CH_3	0.89, d (6.7)	5α, 5*β*, 5*γ*
N-Ac-Ile-6	CO	172.7, C		
	NH		7.03, d (7.6)	6-N-CO
	α	60.1, CH	4.00, dd (7.6, 7.6)	6-N-CO, 6-CO, 6*β*, 6*γ*, 6*β*-CH_3
	β	37.0, CH	1.85, m	
	γ	25.9, CH_2	1.57, m	
			1.21, m	6*β*, 6*δ*, 6*β*-CH_3
	δ	11.4, CH_3	0.90, t (7.4)	6*β*, 6*γ*
	β-CH_3	16.3, CH_3	1.00, d (6.8)	6α, 6*β*, 6*γ*
	N-CO	172.2, C		
	NAc	22.9, CH_3	1.97, s	6-N-CO

[a] ^{13}C NMR data have been obtained from HSQC and HMBC spectra. gHSQC and HMBC readouts. [b] gCOSY readouts. [c] 2C.

boydii metabolism remains unknown, but immunosuppressive activity cannot be ruled out.

Pseudacyclin A (**1**) can be described as cyclo-[*Nδ*-L-Orn^1(*N*^{1}-*N*-acetyl-L-Ile^6)-D-Phe^2-L-Pro^3-L-Ile^4-L-Ile^5]. Noncoded amino acid constituents might indicate the nonribosomal origin of the peptide. The structure contains one *N*-acetylisoleucine and one ornithine, residues that are also conserved in most of the pseudacyclin analogues detected in fermentation broths of a high-producing strain, CBS 119458 (Table 1). Their sequences were deduced from tandem mass spectra. It was found that there was amino acid variability at the fifth and sixth position of the peptide cycloskeleton. Infrared multiphoton dissociation (IRMPD) mass spectra have revealed the side-chain release (Ac-Ile-6 in the case of **1**) as the major dominating fragmentation process (Figure 2). The product ion *m*/*z* 585 originated this way still maintains its cyclic structure (core), resulting in less specific ring-opening mechanisms. Luckily, the sequence-scrambling process[33] is not extensive in pseudacyclins, and two major ring-opening mechanisms dominate in the spectra. They start with primary splitting between amino acid residues 2-1 and 3-2, producing the corresponding $^{x\text{-}y}b_i$ and $^{x\text{-}y}a_i$ ion series. For the fragment ion nomenclature of cyclic peptides see ref 13.

In pseudacyclin B (**2**) the Ile^5 is replaced by an amino acid residue with C_5H_9NO increment, which does not contradict the presence of valine at this position. Formally, pseudacyclin B can be described as [Val^5]PcA (amino acid constituent in square brackets defines the residue change with respect to pseudacyclin A). In pseudacyclin C (**3**) the Ile^6 is methylated. This [Me-acetyl-Ile^6]PcA is the representative with the highest mass of the pseudacyclin family (**3**) and also has the longest retention time on a reversed-phase HPLC column (Figure 3). Pseudacyclin D (**4**) is a co-eluting isobar of pseudacyclin B, having the structure [Val^6]PcA. The co-elution is also observed with pseudacyclins with their protonated molecules at *m*/*z* 712.4401. Only one of them, pseudacyclin E (**5**), [Val^5,Val^6]PcA, is listed in the Experimental Section, but at least two other isobaric analogues were also detected in low amounts. The sequences of the minor pseudacyclins were inferred mostly

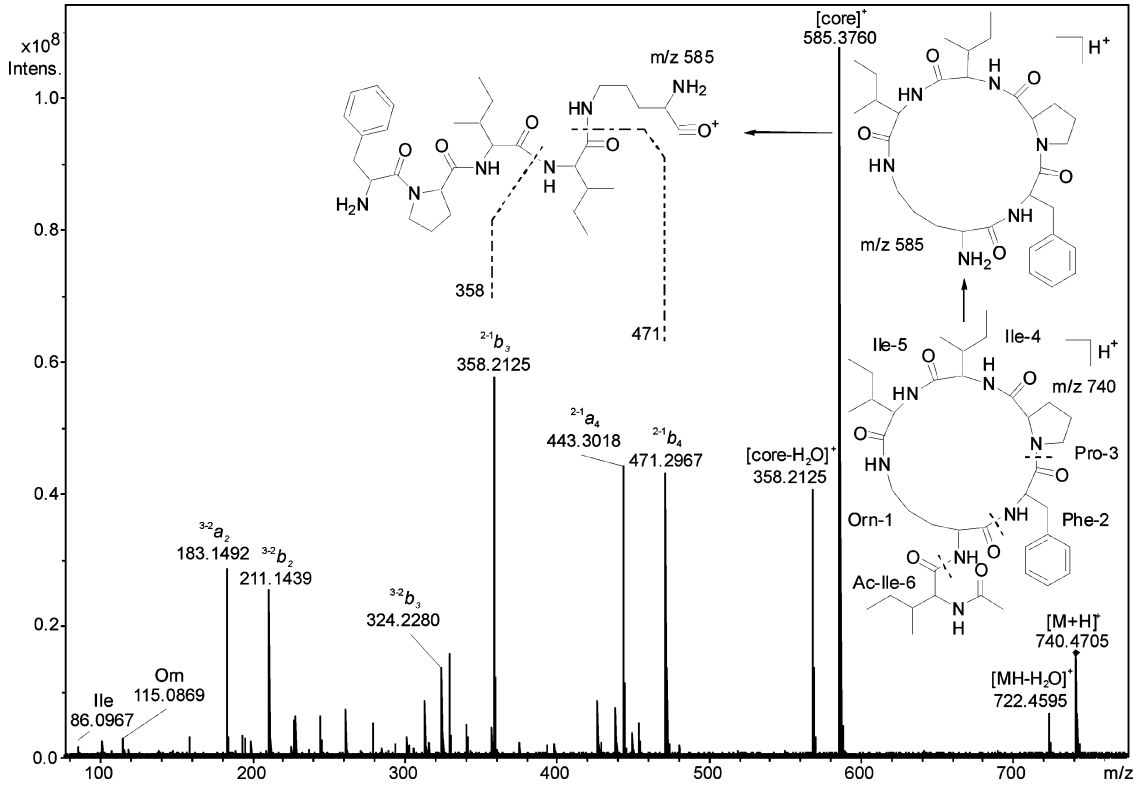


Figure 2. IRMPD mass spectrum of the protonated molecule of pseudacyclin A (**1**). See the text for the description of the fragmentation mechanisms and ion nomenclature. Major ring-opening sites marked.

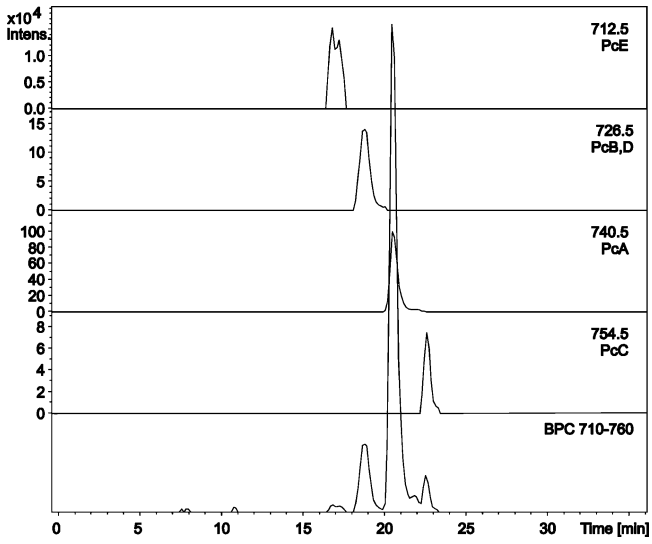


Figure 3. HPLC/MS separation of cyclic peptides. Absolute abundance (*Y* scale) semiquantitatively reflects the peptide ratio. BPC stands for base peak chromatogram reconstructed from the *m*/*z* 710−760 interval (Th).

from high-resolution product ion mass spectra recorded on an FT-ICR mass spectrometer.

Pseudacyclin A has revealed a potential immunosuppressive activity (Experimental Section). On the contrary, only weak NO-synthase inhibition activity and no bactericidal or bacteriostatic effects have been observed.

In addition to the pseudacyclin family, cyclic Phe-Pro was found to be a major constituent in solid-phase extracts of *P. boydii* CBS 119458 culture filtrates assessed by NMR (data not shown). In terms of microorganism specificity and potential diagnostic application, this diketopiperazine is not significant.

Our future work will be focused on the description of novel cyclic peptides that are ubiquitous to most of the strains forming the *P. boydii* complex. Antibodies to these peptides as well as pseudacyclins will represent the basis of a diagnostic ELISA set, which is currently in development. In summary, the current advances in mass spectrometry will lay the experimental foundation for modern sensitive diagnostic tools. We predict that mass imaging of tissues infected by molds will lead to discovery of specific fungal biomarkers. The analysis of intact tissues and bodily fluids will be enabled by ambient mass spectrometric techniques.[34] Single ion detection techniques may beat other instrumental competitors in terms of sensitivity.[35] Innovative molecular approaches together with new modern antifungal drugs then could translate these mass spectrometric advances from bench to bedside.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Jasco DIP-370 polarimeter. UV spectra were measured on a Waters Acquity diode array detector. The IR spectrum was recorded on a Nicolet 205 FT-IR spectrometer. NMR spectra were recorded on a Varian^{UNITY} Inova-400 spectrometer[36] (399.87 MHz for ^{1}H and 100.55 MHz for ^{13}C). The residual solvent signal was used as an internal standard (δ_H 1.93 ppm, δ_C 1.30 ppm). ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC, HMBC, and ROESY spectra were measured using standard manufacturers' software. The ^{1}H NMR spectrum was zero filled to 4-fold data points and multiplied by a window function (two-parameter double-exponential Lorentz−Gauss function) before Fourier transformation to improve the resolution. The ^{13}C NMR spectrum was zero filled to 2-fold data points. Then line broadening (1 Hz) was used to improve the signal-to-noise ratio. Protons were assigned by COSY and TOCSY, and the assignment was transferred to carbons by HSQC. Chemical shifts are given in δ-scale [ppm]; coupling constants are given in Hz. High-resolution mass spectra were measured by FT-ICR on an Apollo II ESI/MALDI ion source in positive-ion mode.[34] IRMPD mass spectra were generated by 0.35 s infrared laser shots (50% attenuation) applied to $[M + H]^+$ precursor ions isolated by a quadrupolar mass filter preceding the ICR cell. Dissociations were also performed in the collisional quadrupole of the instrument. Low-resolution mass spectra

and pseudacyclin A semiquantitation (in SRM mode) were obtained by an ESI-LCQ-DECA ion trap.[36] The parent ion *m/z* 740.5 was selected with a 5 Th window (product ion range scanned between 582 and 588 Th).

Culture Conditions. Fungal strains CBS 119458 (CCF 3082) and CBS 116779 (IHEM 21163, IP 1946.90) were received from Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands, http://www.cbs.knaw.nl/databases/). *P. boydii* CBS 119458 was an isolate from a nasal cavity of a Husky with a chronic rhinitis (Czech Republic). CBS 116779 strain was isolated from a human sinus (China). Genetic and morphological authenticity and purity of the samples were controlled by culturing, rDNA sequencing, and electron and optical microscopy.

Submerged cultivation of both fungal strains has been carried out in 500 mL Erlenmeyer flasks (21 days, 28 °C). Czapek-Dox medium (200 mL) with reduced saccharose content was adjusted to the following optimum composition: $NaNO_3$ (2 g), K_2HPO_4 (1 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4$ (0.01 g), saccharose (10 g), H_2O (pH adjusted to 7.3). The inoculum was prepared by submersed 72 h precultivation. *P. boydii* spores (3×10^7) were added to 150 mL of inoculation media: saccharose (30 g), corn steep (20 g), KH_2PO_4 (2 g), H_2O. The preinoculum was mixed with inoculum in 1:10 ratio (v/v).

Extraction and Isolation. The culture filtrates (200 mL) in 30 Erlenmeyer flasks (500 mL) were extracted twice with CH_2Cl_2 (200 mL). Organic layers were pooled and dried. The dry residue (ca. 20 mg per single Erlenmeyer flask) was reconstituted in MeOH (0.2 mL) and diluted in HPLC solvent A (10× dilution; for composition see below). The resulting solution was injected on a Strata C18E column (1 g, Phenomenex) and desalted by 5% aqueous MeOH. The cartridge was then stepwise eluted with 20% MeOH/H_2O (step 1), 50% MeOH/H_2O (step 2), and 100% (step 3) MeOH. In each step ten 7 mL fractions were collected and evaporated in a speedvac. The fractions were subsequently examined for their metabolite contents. Step 1 (fraction 1) removed the UV-absorbing yellow-to-brownish interfering substances (possibly melanin structures). Diketopiperazines were found in the same step 1 (in fraction 2) by NMR spectroscopy (data not reported). Steps 2 and 3 yielded pseudacyclins. Promising fractions were purified on a reversed-phase Watrex WRP-18 (7 μm) preparative column with gradient elution (A, 5% CH_3CN/H_2O with 0.05% trifluoroacetic acid (TFA); B, 95% CH_3CN/H_2O with 0.05% (TFA), all Merck, gradient grade) at 8 mL/min flow rate and 40 °C. The gradient profile started with a 50 min hold at 0% B and continued with 0−12% B in 5 min; 12−70% B in 150 min, 70−100% B in 25 min, and 20 min hold at 100% B. Separated peptides were detected by UV (214 nm) and subjected to spectroscopic analyses. The total amount of the pure pseudacyclin A obtained from the CBS 119458 strain was 5 mg.

Pseudacyclin A (1): white, amorphous powder; UV (CH_3CN) λ_{max} (log ε) 201 (4.14) nm; IR (Nujol) ν_{max} 1379, 1449, 1525, 1647 cm^{-1}; positive ion HRMS/MS $[M + H]^+$ 740.4705 (calcd for $C_{39}H_{62}N_7O_7$, 740.4705); $[MH - CO]^+$ 712.4761 (calcd for $C_{38}H_{62}N_7O_6$, 712.4745); core 585.3760 (calcd for $C_{31}H_{49}N_6O_5$, 585.3759); $^{2-1}b_4$ 471.2967 (calcd for $C_{26}H_{39}N_4O_4$, 471.2966); $^{2-1}a_4$ 443.3018 (calcd for $C_{25}H_{39}N_4O_3$, 443.3017);$^{2-1}b_3$ 358.2125 (calcd for $C_{20}H_{28}N_3O_3$, 358.2125); $^{3-2}b_3$ 324.2280 (calcd for $C_{17}H_{30}N_3O_3$, 324.2282); 270.1810 (calcd for $C_{13}H_{24}N_3O_3$, 270.1812).

Pseudacyclin B (2): positive-ion HRMS/MS $[M + H]^+$ 726.4549 (calcd for $C_{38}H_{60}N_7O_7$, 726.4549); $[MH - CO]^+$ 698.4594 (calcd for $C_{37}H_{60}N_7O_6$, 698.4600); core 571.3601 (calcd for $C_{30}H_{47}N_6O_5$, 571.3603); $^{2-1}b_4$ 457.2808 (calcd for $C_{25}H_{37}N_4O_4$, 457.2809); $^{2-1}a_4$ 429.2858 (calcd for $C_{24}H_{37}N_4O_3$, 429.2860); $^{2-1}b_3$ 358.2123 (calcd for $C_{20}H_{28}N_3O_3$, 358.2125); $^{3-2}b_3$ 310.2125 (calcd for $C_{16}H_{28}N_3O_3$, 310.2125); 270.1812 (calcd for $C_{13}H_{24}N_3O_3$, 270.1812).

Pseudacyclin C (3): positive-ion HRMS/MS $[M + H]^+$ 754.4858 (calcd for $C_{40}H_{64}N_7O_7$, 754.4862), $[MH - CO]^+$ 726.4903 (calcd for $C_{38}H_{60}N_7O_7$, 726.4549); core 599.3907 (calcd for $C_{32}H_{51}N_6O_5$, 599.3916); $^{2-1}b_4$ 471.2961 (calcd for $C_{26}H_{39}N_4O_4$, 471.2966); $^{2-1}a_4$ 443.3012 (calcd for $C_{25}H_{39}N_4O_3$, 443.3017); $^{2-1}b_3$ 358.2122 (calcd for $C_{20}H_{28}N_3O_3$, 358.2125); $^{3-2}b_3$ 324.2279 (calcd for $C_{17}H_{30}N_3O_3$, 324.2282); 284.1966 (calcd for $C_{14}H_{26}N_3O_3$, 284.1969).

Pseudacyclin D (4): positive-ion HRMS and low-resolution MS/MS $[M + H]^+$ 726.4549 (calcd for $C_{38}H_{60}N_7O_7$, 726.4549); $[MH - CO]^+$ 698.2; core 585.2; $^{2-1}b_4$ 471.1; $^{2-1}a_4$ 443.1; $^{2-1}b_3$ 358.0; $^{3-2}b_3$ 324.2.

Pseudacyclin E (5): positive-ion MS/MS and low-resolution MS/MS $[M + H]^+$ 712.4401 (calcd for $C_{37}H_{58}N_7O_7$, 712.4392); $[MH - CO]^+$ 684.4; core 571.2; $^{2-1}b_4$ 457.1; $^{2-1}a_4$ 429.1; $^{2-1}b_3$ 358.2; $^{3-2}b_3$ 310.0.

Evaluation of Biological Activities of Pseudacyclin A. Cytotoxic and antiproliferative activities of pseudacyclin A were analyzed on resting and mitogen-activated (5 μg/mL of concanavalin A, con A) human lymphocytes.[37] IC_{50} cytotoxicity values 32.0 ± 6.8 and 12.9 ± 4.2 μM were determined for resting and con A-activated lymphocytes, respectively. Preferential toxicity of pseudacyclin A to activated, but not resting, lymphocytes indicates a potential immunosuppressive activity of this metabolite. By contrast, pseudacyclin A induced only a weak inhibition of NO-synthase activity[38] measured as 24 h production of NO_2^- by polysaccharide (0.5 μg/mL)-activated B10R macrophages with an IC_{50} of 41.4 ± 10.4 μM. Antimicrobial activity of pseudacyclin A has been evaluated against the following set of standard reference strains: *S. aureus, E. faecalis, E. coli, P. aerugonosa, S. hemolyticus, C. albicans, C. crusei, C. tropicalis, C. parapsilosis*. No bactericidal or bacteriostatic effect has been observed.

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Supporting Information Available: ^1H, HMBC, and HSQC NMR spectra for compound **1** and SEM images of a surface cultivation of *P. boydii* are provided free of charge via the Internet at http://pubs.acs.org.

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