A Cell Wall-Active Lipopeptide from the Fungus *Pochonia bulbillosa*

Frank E. Koehn,*[,†] Donald R. Kirsch,[‡] Xidong Feng,[†] Jeffrey Janso,[†] and Mairead Young[†]

Natural Products Discovery and Discovery Analytical Chemistry, Chemical and Screening Sciences, Wyeth Research, Pearl River, New York 10965, and Cambria Biosciences, Woburn, Massachusetts 01801

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Bioassay-directed fractionation of a fermentation of *Pochonia bulbinosa,* culture 38G272, led to the isolation of a series of structurally novel, prospective cell wall-active lipopeptides. The main component of this suite is **1**, a linear hexapeptide with a δ-hydroxymyristic acid amide substituted N-terminus. The structure was deduced using high-field microsample NMR, Fourier transform mass spectrometry, and microscale chemical degradation. The potent cell wall activity and synthetically accessible structure of **1** make it a potential lead for further investigation.

Fungal pathogens are eukaryotes and show significant biochemical similarity to their hosts. Therefore, a major prerequisite for the discovery of nontoxic antifungal therapeutics is the identification of novel fungal-selective biochemical targets and compounds that selectively inhibit these targets. Fungal cell wall biosynthesis has been an area of long-term interest, since the production of a cell wall is essential for fungal viability and human cells lack both a cell wall and the enzymes that synthesize the fungal cell wall polysaccharide components.[1] Our efforts to identify novel antifungal cell wall-acting agents have in part focused upon the use of an assay designed to identify general inhibitors of cell wall biosynthesis.[2] This assay exploits a *Neurospora crassa* os-1 mutation, which allows cell growth in the absence of a cell wall.[3] In the presence of concentrations of cell wall-acting antifungal agents that inhibit the growth of wild-type strains, strains carrying an os-1 mutation will grow as wall-less protoplast cells. Most compounds acting on non-cell wall targets have nearly identical inhibitory actions on os-1 and wild-type strains, and thus differential growth with the production of protoplasts can be used to identify compounds with this desired mechanism of action. We have reported the identification of a novel cyclic lipopeptide with this screen: 15G256γ.[4] This compound showed antifungal activity and induced the production of protoplasts in a number of fungal species, yet inhibited neither chitin synthase nor β-1,3-glucan synthetase, and may thus be acting through a cell wall biosynthesis regulatory mechanism.[5] The interesting mechanism and challenging structure of this molecule prompted us to pursue additional screening in order to identify synthetically accessible molecules that could serve as the basis for medicinal chemistry optimization.

Fungi of the genus *Pochonia* sp. are producers of resorcylic acid lactone metabolites such as pochonins, monocillins, radicicol, and various analogues, and they also produce the unique heterospirocycle pseurotin A.[6,7] These compounds show antiviral and antiparasitic activity and HSP90 inhibition.[8] The broad biosynthetic capabilities of this genus along with our interest in simple antifungal natural products that might serve as medicinal chemistry leads prompted us to examine in detail a culture of *Pochonia bulbillosa*.

The *n*-butanol broth extract of a strongly os-1-active 1 L fermentation of culture 38G272 was chromatographed on a column of LH-20 in MeOH, and the subsequent active fractions were subjected to repeated semipreparative reversed-phase HPLC in MeOH/H_2O/TFA and then MeCN/H_2O/TFA mixtures. MS and ^{1}H NMR examination of active fractions showed that the os-1 activity was due to a complex suite of lipopeptides ranging in molecular weight from 700 amu to over 1200 amu. Repeated reversed-phase HPLC furnished 0.7 mg of the major component **1** (Chart 1), which gave a 20 mm zone of protoplast production in the os-1 assay at 600 ng per disk. Since **1** was the most abundant of the active components, we focused our attention on its structure elucidation.

The low-resolution positive ion ESMS of **1** gave a nominal mass of *m*/*z* 821 for the M + H, and high-resolution positive ion nanoelectrospray FTMS showed a sodium adduct of the molecular ion at *m*/*z* 843.48364 $[M + Na]^+$ in positive mode and a deprotonated molecular ion at *m*/*z* 819.48767 $[M - H]^-$ in negative mode, corresponding to a molecular formula of $C_{41}H_{68}N_6O_{11}$ (Δ mmu = −0.19, 0.34, respectively). The ^{1}H NMR spectrum in DMSO-d_6, first at 300 MHz and then at 500 MHz, displayed a number of distinctive features. Among these were resonances for six NH amide protons (determined by methanol-d_4 exchange), four of which were contained in a strongly overlapped multiplet at δ 7.85, a phenolic OH at δ 9.13, six methyl doublets clustered in groups of three at δ 0.9 and 1.1, an overlapping muliplet of α-protons centered at δ 4.1, and a large envelope of CH_2 protons at δ 1.2. These features, taken together with the molecular formula, suggested the molecule was a hexapeptide consisting of single residues of threonine, valine, and tyrosine, three alanine residues, and an oxygenated aliphatic C-14 acid or amide unit. The strongly overlapping alanine signals and the molecular formula taken together with the general lack of ^{1}H NMR spectral dispersion suggested a linear molecule.

The 500 MHz 2D TOCSY and multiplicity-edited HMQC data confirmed the presence and allowed assignment of the constituent amino acid residues as listed in Table 1. Readily assigned via 2D TOCSY experiments were the ^{1}H spin systems for the threonine at δ 7.87 (NH), 4.18 (Hα), 3.74 (Hβ), and 1.07 (Hγ), the tyrosine at δ 7.99 (NH), 4.61 (Hα), and 2.89, 2.61 (Hβ), the aromatic protons at δ 7.03 and 6.61, and the valine residue at δ 8.18 (NH), 4.15 (Hα), 2.03 (Hβ), and 0.829, 0.83 (Hγ). The three individual alanine spin systems showed considerable overlap in the 1D and 2D homonuclear spectra, with resonances assignable for alanine-1 at δ 8.26 (NH), 4.17 (Hα), and 1.21 (Hβ), alanine-2 at δ 7.87 (NH), 4.22 (Hα), and 1.14 (Hβ), and alanine-3 at δ 7.81 (NH), 4.24 (Hα), and 0.98 (Hβ). The 2D TOCSY spectrum contained additional cross-peaks showing coupling of a proton at δ 3.31 to a methylene at δ 1.59, 1.45 and a methylene at δ 2.11; the latter was assumed to be adjacent to the amide carbonyl by virtue of proton chemical shift. The δ 3.31 was also coupled into the methylene envelope at δ 1.28. These data indicated the presence of a hydroxy group located somewhere along the C-14 chain. ^{13}C NMR chemical shifts were assigned by means of a multiplicity-edited HMQC experiment and are listed in Table 1.

We turned to g-HMBC and ROESY spectra to provide sequence information. The carbonyl region of the g-HMBC spectrum

* To whom correspondence should be addressed. Tel: 845-602-4094. Fax: 845-602-4094. E-mail: koehnf@wyeth.com.
† Wyeth Research.
‡ Cambria Biosciences.

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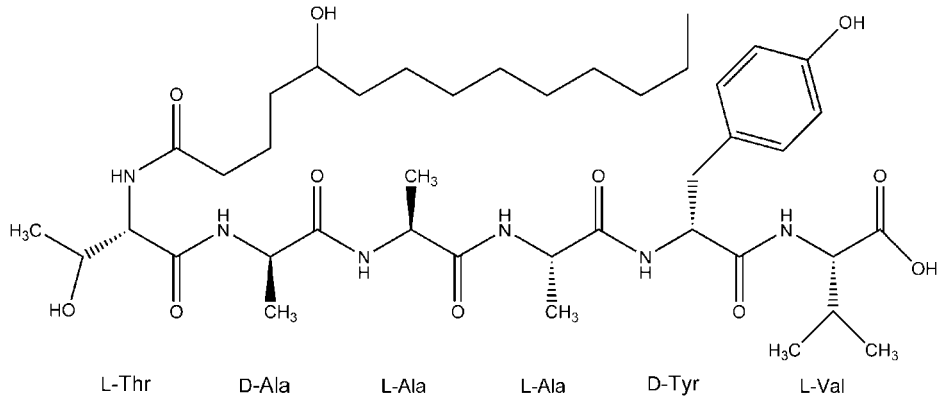
Chart 1



1

Table 1. ^{1}H (500 MHz) and ^{13}C (75 MHz) NMR Data for Compound **1** in DMSO-d_6 at 30 °C[a]

residue	^{13}C (75 MHz)	^{1}H (500 MHz, mult, *J* in Hz)	HMBC correlation
Thr			
C═O	170.6		
NH		7.87 (d, 1H, *J* = 8.7)	172.1
α	59.19	4.18	170.6, 67.2
β	67.43	3.74 (q, 1H, *J* = 7.4)	170.6
γ	20.28	1.07 (d, 3H, *J* = 6.7)	67.2, 58.2
Ala 1			
C═O	171.6		
NH		8.26 (d, 1H, *J* = 7.3)	170.6
α	57.0	4.17 (m, 1H)	170.6
β	17.52	1.21 (d, 3H, *J* = 7.2)	171.6, 48.5
Ala 2			
C═O	171.2		
NH		7.87 (d, 1H, *J* = 7.2)	
α	48.47*	4.22 (m, 1H)	
β	17.77	1.14 (d, 3H, *J* = 6.7)	171.2, 48.0
Ala 3			
C═O	171.2		
NH		7.81 (d, 1H, *J* = 7.4)	171.3
α	47.90*	4.24 (m, 1H)	171.6
β	18.30	0.98 (d, 3H, *J* = 6.7)	171.6, 48.0
Tyr			
C═O	171.31		
NH		7.99 (d, 1H, *J* = 8.9)	171.7
α	53.81	4.61	171.2, 38.2
β	38.15	2.89 (dd 1H, *J* =15.0, 4.0)	171.2, 127.6, 130.1
		2.61 (dd, 1H, *J* = 15.0, 11.0)	171.2, 127.6,130.1
other		7.03, (dt, 2H, *J* = 8.4)	38.15, 114.6, 130.1
		6.61 (dt, 2H, *J* = 8.4)	38.15, 114.6, 130.1
		9.13 (OH)	
Val			
C═O	172.8		
NH		8.18 (br, 1H)	171.2
α	57.0	4.15 (br, 1H))	
β	30.0	2.03 (m, 1H)	172.2, 56.99
γ	19.0	0.83 (d, 3H, *J* = 6.7)	30.04, 56.99
	19.0	0.83 (d, 3H, *J* = 6.7)	
HMA			
C═O	172.2		
α	35.10	2.11 (m, 2H)	
β	21.6	1.60 (m, 2H)	
γ	36.5	1.24 (m, 2H)	
δ	69.2	3.31 (m, 1H)	
e	37.2	1.27 (m, 2H)	
ϕ	25.2	1.24 (m, 2H)	
C8−C13	29.8−22.7	1.24 (br envelope)	
C14	13.9	0.86 (t, 3H)	

[a] HMA: *δ*-hydroxymyrstic acid amide. *Assignment may be interchanged due to overlap.

contained limited information for linking the residues, with the carbonyl signals for all three alanine moieties and the tyrosine all located within 0.5 ppm of *δ* 171.3. Correlation peaks from Hα and H*β* in the C14 unit at *δ* 2.11/172.3 and *δ* 1.59,1.45/172.3, respectively, allowed assignment of the N-terminal amide carbonyl chemical shift. The remaining correlations in the carbonyl region of the HMBC spectrum were intraresidue NH/C═O for tyrosine at *δ* 7.99/171.7 and alanine-3 at *δ* 7.81/171.3. An additional signal at

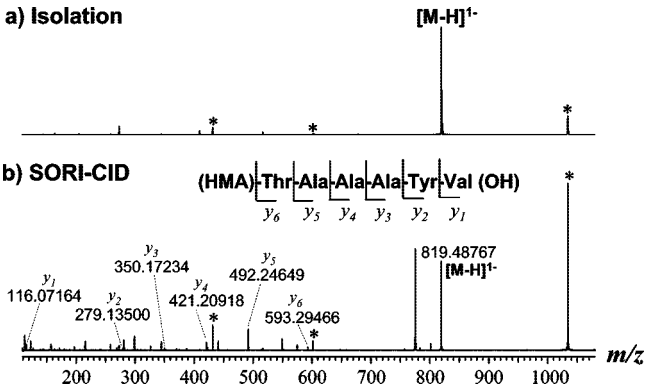


Figure 1. Negative mode nanoelectrospray FTMS mass spectrum of **1**. (a) Multi-CHEF isolation of the $[M - H]^-$ precursor ion. (b) Multi-CHEF SORI-CID mass spectrum of the molecular ion $[M - H]^-$ with inset showing the sequence coverage. Internal reference peaks are labeled with stars [*] at the predicted values of *m/z* 431.98233, 601.97898, and 1033.98812 from Agilent ES tuning mix. HMA: hydroxymyristic acid amide substituted N-terminus.

δ 3.31/69.2 in the multiplicity-edited HSQC was assigned to an OH-bearing carbon located along the chain of the N-terminal C-14 amide. The remaining peaks in the HMBC spectrum confirmed the intraresidue assignments made by TOCSY and HSQC (Table 1). The 2D ROESY spectrum contained additional information to allow partial sequence assignment. The presence of the C-14 amide on the threonine nitrogen could be ascertained by a ROESY cross-peak from the threonine NH at δ 7.87 to the Hα methylene at δ 2.11. Furthermore, the tyrosine NH at δ 7.99 showed a ROESY cross-peak to one alanine Hα at δ 4.23, and the tyrosine aromatic protons showed ROESY cross-peaks to the Hβ methyl group of Ala-1 as well, suggesting an Ala-Tyr partial structure.

The position of the OH group in the C-14 chain was assigned to the γ-position by using 2D TOCSY and HSQC-TOCSY experiments at 20, 55, and 70 ms mixing times to establish the proton and ^{13}C NMR chemical shifts of methylene units in stepwise fashion out from the OH group. At a mixing time of 20 ms, correlations were observed from protons at δ 3.31 to 1.27 to ^{13}C NMR signals at δ 37.2 and 36.5, indicating the directly adjacent vicinal protons and associated carbons. Increasing the mixing time to 55 ms and then to 70 ms showed additional stepwise increasing correlations to δ 1.59/21.6 and then to δ 2.11/35.1, indicating these methylene units were located at successively removed positions. Previous assignment of the δ 2.11 signal to the α-position by g-HMBC therefore placed the OH at the δ-position. Comparison of observed and calculated ^{13}C NMR chemical shifts agreed closely with the δ OH assignment.

Since NMR methods did not yield sufficient information for the complete sequence assignment, we resorted to FTMS.[9] The negative mode nanoelectrospray multi-CHEF SORI-CID mass spectra of **1** showed all y sequence ions necessary to complete the sequence assignment (Figure 1, Table 2). The positive mode IRMPD mass spectrum of the $[M + Na]^+$ molecular ion provided equally thorough sequence information (Supporting Information).

Acid hydrolysis followed by derivatization to produce the *n*-propyl pentafluoropropionyl esters and chiral GC analysis showed that the peptide portion of the molecule consisted of one residue each of D-Tyr, L-Val, L-Thr, and D-Ala and two residues of L-Ala. Given three alanine units, the question remained as to the sequence-specific configuration assignment. The linear nature of the molecule and lack of dispersion of the NMR spectrum, particularly the alanine CHα and methyl resonances, precluded reliable assignment by NMR. Therefore we resorted to microscale chemical degradation methods with the goal of generating alanine-bearing peptides that could be chromatographically compared with authentic standards. Under consideration of the work of Anderson et al., it was hypothesized that given the peptide sequence, differential rates of hydrolysis under mild acid hydrolysis conditions should liberate the tripeptide Ala-Ala-Ala as an early product in the hydrolysis.[10] Compound **1** was subjected to mild acid hydrolysis in 2:1 MeCN/1 N HCl·H_2O, and the reaction mixture was monitored by LCMS. After 3 h the mixture showed the presence of the diagnostic D-Ala-L-Ala-L-Ala tripeptide with $[M + H]^+$ $m/z = 232$, by comparison with an authentic standard. Fragment partial hydrolysis products were also observed for $[M + H]^+$ $m/z = 595$, 496, 352, and 333 for TAAAYV,TAAAY, AYV, and TAAA, respectively. These results complete the sequence assignment and structure elucidation.

Recent efforts to develop novel, clinically useful antifungal agents have focused on compounds of the echinocandin class.[11] These lipopeptide compounds prevent fungal cell wall glucan synthesis by inhibiting the enzyme β-1,3-D-glucan synthase, a highly selective target since mammals do not synthesize β-1,3-D-glucans, providing such compounds with high intrinsic potential for selectivity. The clinically employed echinocandins are based upon natural product leads optimized via semisynthetic chemical approaches. As an example, caspofungin, the first clinically approved agent in this class, is an aza-substituted bisamine derivative of the antibiotic pneumocandin B_0.[12] While several echinocandins have clinically useful antifungal potency and spectrum (e.g., caspofungin, micofungin, and anidulafungin), their poor absorption after oral administration limits their dosing to the intravenous route.[13,14] The unattractive pharmacological properties of these compounds coupled with significant limitations for synthetic modification have stimulated the search for alternate structural types that will inhibit cell wall synthesis.[1] The putative cell wall synthesis inhibitory activity of **1** coupled with its potential for synthetic elaboration could make this compound a valuable anti-infective lead.

Experimental Section

General Experimental Procedures. HPLC analysis was performed on an HPLC system from Agilent Technologies (Wilmington, DE) consisting of an Agilent 1100 pump with an online degasser, a diode-array detector (which was set at a 215 nm wavelength), and an autosampler. For instrument control, data acquisition, and processing an Agilent Chemstation data system was applied (Agilent Chemstation B.1.03).

NMR experiments were performed on a Bruker DRX 500 equipped with a 5 mm TXI Cryoprobe, or a Bruker AMX 300 spectrometer. The HRMS spectra of lipopeptide 38G272-820 were measured using a Bruker Daltonics APEXII FTICR mass spectrometer equipped with an actively shielded 9.4 T magnet, an external Bruker APOLLO ESI source, and a Synrad 50W CO_2 CW laser. Typically, 5 μL samples were loaded into the nanoelectrospray tip, and a high voltage, about 800 V, was applied between the nanoelectrospray tip and the capillary. Data reported here are based on internal calibration using a HP tuning mix. The ESI-FTMS/MS experiments were conducted on either the deprotonated molecular ion at *m/z* 819 for negative mode or the sodium adduct molecular ion at *m/z* 843 for positive mode, using sustained off-resonance irradiation with collision-induced dissociation (SORI-CID) or an infrared multi-photon dissociation (IRMPD). Data reported here are from SORI-CID experiments where the precursor ion of *m/z* 819 was isolated together with three other HP tuning mix ions (predicted values of *m/z* 431.98233, 601.97898, and 1033.98812) using a multi-CHEF (correlated harmonic excitation fields) sweep (Figure 1). The parent ions were activated by an rf pulse, off resonance from the parent ions by 1000 Hz. Argon gas was pulsed into the ICR cell to collide with the activated parent ions to yield fragment ions. The SORI-CID mass spectrum was internally calibrated using HP tuning mix peaks specified above.

Fungal Material. *P. bulbillosa* 38G272 was isolated from unknown material collected from oak forests in San Jose Province, Costa Rica, in 1992. The nuclear internal transcribed spacer region (ITS) of 38G272 (Cyan308) was searched against the GenBank database via BLASTN 2.2.1[15] and found to be 100% similar to *P. bulbillosa*[16] (CBS 145.70). In a study comparing ITS sequences of *Verticillium* sect. *Prostrata*, Zare et al.[17] found that *Verticillium bulbillosum* formed a clade with

Table 2. Proposed Analysis of FTMS/MS of *m/z* 819.48767 [(HMA)TAAAYV(OH) − H]$^{-a}$

exptl mass	pred mass	Δ (mmu)^b	rel abun	elemental formula	structure assignments	ion assignments
819.48767	819.48733	0.34	87%	$C_{41}H_{67}N_6O_{11}^-$	$[M - H]^-$	precursor
593.29466	593.29405	0.61	3%	$C_{27}H_{41}N_6O_9^-$	$[TAAAYV(OH)]^-$	y_6
492.24649	492.24637	0.12	22%	$C_{23}H_{34}N_5O_7^-$	$[AAAYV(OH)]^-$	y_5
421.20918	421.20926	−0.08	8%	$C_{20}H_{29}N_4O_6^-$	$[AAYV(OH)^-$	y_4
350.17234	350.17214	0.20	2%	$C_{17}H_{24}N_3O_5^-$	$[AYV(OH)]^-$	y_3
279.13500	279.13503	−0.03	2%	$C_{14}H_{19}N_2O_4^-$	$[YV(OH)]^-$	y_2
116.07164	116.07170	−0.06	6%	$C_5H_{10}NO_2^-$	$[V(OH)]^-$	y_1

[a] HMA: ξ-hydroxymyristic acid amide substituted N-terminus. [b] Errors (mmu) calculated as experimental value − predicted value.

other *Verticillium* spp., which parasitize nematode eggs or cysts and form dictyochlamydospores. *V.* sect. *Prostrata* was revised at the generic level,[18] resulting in the renaming of *V. bulbillosum* to *Pochonia bulbillosa.*[18] The ITS sequence for fungus 38G272 was deposited in GenBank under accession number EU999952.

Culture 38G272 was grown on cornmeal agar (Oxoid) at room temperature (about 24 °C) for morphological analyses. The agar-grown culture was examined microscopically on the seventh and 24th days of incubation. By the seventh day of growth, lunate or crescent-shaped conidia were visible on the aerial mycelia in slimy heads. Conidia were produced by solitary phialides or 2−3 phialides per node. By the 24th day of growth, thick-walled dictychlamydospores were present. The morphology of 38G272 concurs with the ITS identification of 38G272 as *P. bulbillosa*. Fermentation of 38G272 for chemical studies was carried out in 1 L shake flasks in modified Sabouraud dextrose broth, substituting maltose for dextrose.

Extraction and Isolation of 1. The whole fermentation broth from a 1 L fermentation of culture 38G272 was centrifuged, the pellet removed, and the supernatant extracted twice with equal volumes of *n*-BuOH. A small portion of the crude BuOH-soluble material was chromatographed on a 15 cm Rainin microsorb C_{18} HPLC column with 60% 0.1 N TFA/MeCN, and fractions were assayed for os-1 activity, which appeared to elute in a broad peak spanning 4−15 min. On the basis of this result the bulk of the crude BuOH-soluble material was loaded onto a 25 mm × 60 cm column of Sephadex LH-20 and eluted with MeOH in 10 mL fractions. The main peak of os-1 activity eluted in fractions 10 through 18, which were combined (36 mg) and subsequently chromatographed on a 4.6 mm × 15 cm Rainin C_{18} microsorb column with 67% MeOH/0.1 N TFA. Compounds that failed to elute were washed from the column with MeOH to give 5.9 mg of material, which by HPLC showed the presence of **1** as a major component. This material was dissolved in MeOH and chromatographed on a 1.0 cm × 25 cm Rainin microsorb C_{18} column in 71% MeOH/0.1 N TFA. Fractions were collected on the basis of absorbance at 225 nm, giving 0.7 mg of **1** along with smaller amounts of related compounds.

Determination of Amino Acid Configuration. Approximately 10 μg of **1** was dissolved in 6 N constant boiling HCl and heated at 110 °C for 22 h. The solution was diluted with H_2O and lyophilized. The residue was dissolved in HCl-1-propanol (prepared by additional of acetyl chloride, 10%, to 1-propanol) and heated at 90 °C for 1 h and then evaporated under an N_2 stream. The residue was taken up in 10% pentafluoropropionic anhydride in CH_2Cl_2 and heated at 50−60 °C for 4 h. The solvent was removed under an N_2 stream and the residue redissolved in CH_2Cl_2 and analyzed by GC-MS with selective ion monitoring on a Chirasil capillary column. Comparison and co-injection with *n*-propyl pentafluoropropionyl derivatives of authentic L and D amino acid standards showed the hydrolysis product to be composed of D-Tyr, L-Val, L-Thr, D-Ala, and L-Ala. The D,L-Ala components were present in a 1:2 ratio, respectively.

Partial Hydrolysis of 1: Determination of Sequence Position of D- and L-Alanine. A UV-HPLC method to separate the three forms of the alanine tripeptides DLL, LDL, and LLD was developed using the following chromatographic conditions: a YMC PackPro AM (5 μm, 150 mm × 4.6 mm i.d.) column from Waters (Milford, MA); mobile phase composed of (A) 0.02% TFA in H_2O and (B) 0.02% TFA in MeCN. Gradient elution was performed as follows: 0−6 min 5% B, 6−10 min 5−95% B, and 10−15 min 95% B at a flow rate of 1 mL/min. The retention of authentic alanine tripeptide standards (Bachem) were as follows: D-Ala-L-Ala-L-Ala at 2.5 min, L-Ala-L-Ala-D-Ala at 3.1 min, and L-Ala-D-Ala-L-Ala at 4.2 min. For analysis, a sample of **1** (40 μg) was dissolved in 1.0 mL of MeCN/1 N HCl H_2O (3:2) in a sealed vial at 30 °C and monitored by LCMS with single-ion and full TIC detection. After 24 h no significant products were observed and the reaction mixture was heated to 90 °C. LCMS using absorbance at 215 nm single-ion monitoring at $m/z = 232$ showed the presence of D-Ala-L-Ala-L-Ala ($t_R = 2.44$ min). Additional major products observed during the hydrolysis were $[M + H]^+$ $m/z = 595$, 496, 352, and 333 for TAAAYV, TAAAY, AYV, and TAAA, respectively. Minor amounts of presumably racemized trialanine were also observed.

Antifungal Assays. Assays were performed as described in ref 2. Briefly, os-1 strain spores were inoculated into growth media and test samples applied to the agar surface. The assay plates were scored after 36−48 h of incubation at 37 °C. Active samples were identified by the appearance of characteristic growth zones surrounding the sample. Activity was confirmed by microscopic examination of the activity zones to determine whether mycelial growth (inactive) or protoplasts (active) were present.

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Note Added after ASAP Publication: The structure of compound **1** was corrected.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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