Serinocyclins A and B, Cyclic Heptapeptides from *Metarhizium anisopliae*

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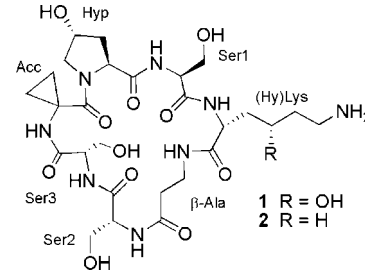
Two new cyclic heptapeptides, serinocyclins A (**1**) and B (**2**), were isolated from conidia of the entomopathogenic fungus *Metarhizium anisopliae*. Structures were elucidated by a combination of mass spectrometric, NMR, and X-ray diffraction techniques. Serinocyclin A (**1**) contains three serine units, a hydroxyproline (Hyp), a β-alanine (β-Ala), and two uncommon nonproteinogenic amino acids, 1-aminocyclopropane-1-carboxylic acid (Acc) and γ-hydroxylysine (HyLys). The peptide sequence established for **1** by NMR is cyclo-(Acc-Hyp-Ser1-HyLys-β-Ala-Ser2-Ser3). Serinocyclin B (**2**) has Lys in place of the HyLys unit found in **1**. Chiral amino acid analysis indicated the presence in both compounds of one (2*S*,4*R*)-Hyp, two L-Ser, and one D-Ser residue. A Lys found in the hydrolyzate of **2** was established as D-configured. A crystal structure of **1** established the position of the D-Ser (Ser2) and the absolute configuration of the HyLys unit (2*R*,4*S*). The absence of methyl groups is unusual among fungal peptides and, along with the charged lysyl side chain and multiple hydroxyl groups, contributes to the polar nature of the compounds. Serinocyclin A produced a sublethal locomotory defect in mosquito larvae at an EC_{50} of 59 ppm.

Metarhizium anisopliae (Metschnikoff) Sorokin (Clavicipitaceae) ranks prominently among entomopathogenic fungi used commercially for biological control of insect pests.[1,2] Consequently, considerable attention has been focused on the basic biology of this fungus with intense interest in identifying factors affecting its pathogenicity to its hosts,[3] including the possible role of secondary metabolites as virulence determinants.[4,5]

The best-known secondary metabolites from *M. anisopliae*, the depsipeptidal destruxins,[6] exhibit a variety of acute toxic effects against insects[7] as well as more subtle physiological effects thought to impact insect immunity.[8,9] Recently, we reported the production of NG-391 (and the related NG-393) by this fungus and demonstrated its mutagenic activity.[10] To date, no definitive evidence has been presented demonstrating the adaptive significance of destruxins, NG-391, or any of the other known secondary metabolites of *M. anisopliae* referenced in our prior report.[10] The nonproteinogenic amino- and hydroxy-acid constituents found in the destruxins and NG-39x compounds are hallmarks of nonribosomal peptides (NRPs) and indicate the involvement in their biosynthesis of nonribosomal peptide synthetases, large multifunctional enzyme complexes that recognize and activate amino acids (or hydroxy-acids) and incorporate them into a peptide (or depsipeptide) chain.[11] In NG-39x and related compounds, a single amino acid is incorporated into a hybrid product of a nonribosomal peptide synthetase and a polyketide synthase.[12,13] Genome sequencing studies have revealed that individual fungal species have the biosynthetic capacity to produce many more NRPs than have been chemically characterized.[14] Previously, we reported the isolation of mutants of *M. anisopliae* in which a nonribosomal peptide synthetase gene of unknown function had been inactivated by targeted gene disruption.[15] This study led to a comparison of extracts in which novel peptides were detected in a wild-type strain (ARSEF #2575), but not in mutants in which the nonribosomal peptide synthetase gene had been disrupted. Here, we present the structural characterization of the two major representatives of this new peptide class from *M. anisopliae*, which we have named serinocyclins A (**1**) and B (**2**). We also report a sublethal toxic effect of **1** on mosquito larvae.

Chart 1



Results and Discussion

Low-resolution ESIMS analysis of crude methanolic extracts of conidia of *M. anisopliae* var. *anisopliae* (ARSEF #2575) showed signals from pseudomolecular ions indicating compounds with molecular weights of 672 and 656. A strain of *M. anisopliae* var. *acridum* Driver & Milner (Clavicipitaceae) derived from the commercial biological control product Green Muscle (hereinafter referred to as "GMD") produced 5–10 times more of these compounds than ARSEF #2575 and, consequently, was used as a source of material for their isolation and characterization. The target compounds eluted together on Sephadex LH-20. Preparative HPLC followed by ESIMS analysis of fractions revealed a family of related compounds with two major components that were readily isolated to yield **1** and **2** of high purity. Their structures were elucidated using ESIMS and NMR spectroscopy, chiral amino acid analysis, and X-ray crystallography.

HRESIMS data for **1** were consistent with the molecular formula $C_{27}H_{44}N_8O_{12}$. Following deuterium exchange in CH_3OD, ESIMS experiments indicated the presence of 13 exchangeable protons. ^{1}H NNMR spectra showed 37 protons belonging to **1**, including six with signals in the region between 7.4 and 9.5 ppm that underwent solvent exchange in CD_3OD, suggesting that they were amides. This left seven exchangeable protons unassigned. ^{13}C NMR and DEPT135 experiments indicated the presence of 27 carbons, including seven carbonyl signals and 20 sp^3 carbons. The spectra showed seven methines including two downfield signals at δ_C 66.8

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Table 1. NMR Spectroscopic Data (CD_3OH) for Serinocyclin A (**1**)

unit[a]	position	δ_C[b] (or δ_N[c]), mult	δ_H[d] (J in Hz) [$-\Delta\delta/\Delta T$ ppb/K]	HMBC[e]	ROESY[f]
Acc	NH	125.7, NH	9.50, br s [5.9]	C, α, β2[g]; Ser3 C[g]	β1b, β2a
(1)	α	37.5, qC			
	β1a (*pro-R*)	13.5, CH_2	1.60, ddd (10.4, 7.8, 4.9)	C, N, α, β2	HyLys NH, β1 δ1 ϵ; β-Ala NH, β2
	β1b (*pro-S*)		0.73, ddd (10.4, 7.4, 5.0)	C[g], N, α, β2	HyLys NH; β-Ala NH, β2
	β2a (*pro-R*)	12.9, CH_2	1.33, ddd (9.7, 7.6, 5.7)	C[g], N, α, β1	Hyp δ2
	β2b (*pro-S*)		1.04, ddd (10.4, 7.2, 5.6)	C, N, α, β1	Hyp δ2
	C═O	174.7, qC			
Hyp	N	129.1, tN			
(2)	α	62.3, CH	4.45, m	C, N; Acc C	β**1**,2
	β1 (*pro-R*)	38.8, CH_2	2.32, m	N, γ, δ	
	β2 (*pro-S*)		1.94, m	C, N, α	δ1, Ser1 NH
	γ	71.1, CH	4.49, m	α, β, δ	β1,**2**, δ**1**,2
	δ1 (*pro-S*)	57.8, CH_2	4.04, m	Acc C	Ser1 NH
	δ2 (*pro-R*)		3.73, m	α, β, γ; Acc C	Acc β2a,**b**
	C═O	175.5, qC			
Ser1	NH	109.6, NH	7.54, d (8.0) [2.1]	α, β; Hyp C	β**1**,2; Hyp-β2, δ1
(3)	α	58.1, CH	4.42, m	C, N, β	
	β1 (*pro-S*)	63.2, CH_2	4.22, dd (12.0, 5.5)	C, N, α	NH; HyLys NH; β-Ala NH; Ser3 NH
	β2 (*pro-R*)		3.83, m	C, N, α	
	C═O	172.8, qC			
HyLys	NH	119.7, NH	7.49, d, (7.9) [1.1]	α, β; Ser1 C	β1; Acc β1**a**b, Ser1 β1, β-Ala NH
(4)	α	53.1, CH	4.60, ddd (10.8, 7.8,2.5)	C, N, β, γ[g]; Ser1 C, β-Ala N[g]	β1,2
	β1	40.1, CH_2	2.04, m	C, N, γ	Acc β1a
	β2		1.98, m	C, N, γ	
	γ	66.8, CH	3.81, m		
	δ1	35.8, CH_2	1.85 dddd (13.7. 6.9, 6.8, 3.7)	β, γ, ε, ε-N	Acc β1a
	δ2		1.71, m	β, γ, ε	
	ε	38.9, CH_2	3.07, m	γ, δ, ε-N	Acc β1a
	ε-NH_3	31.0, NH_3^+		δ**1**,2	
	C═O	174.8, qC			
β-Ala	NH	113.8, NH	7.99, m [4.2]	β; HyLys C	α1,**2**, β1,**2**; Acc β1ab, Ser1 β1, HyLys NH
(5)	β1	37.6, CH_2	3.73, m	C, N, α; HyLys C[g]	
	β2		3.06, m	α, HyLys C	Acc β1ab
	α1	37.0, CH_2	2.38, ddd (8.0, 6.8, 1.6)	C, N, β	Ser2 NH
	α2		2.28, m	C[g], N, β; Ser2 N	
	C═O	175.2, qC			
Ser2	NH	122.6, NH	8.46, d (8.8) [6.9]	α; β-Ala C	β1,**2**; β-Ala α1
(6)	α	55.9, CH	4.44, m	C, N[g]	
	β1 (*pro-R*)	61.2, CH_2	3.86, m	C, N, α	
	β2 (*pro-S*)		3.75, m	C, N	
	C═O	172.2, qC			
Ser3	NH	115.2, NH	7.97, m [4.6]	C, α;Ser2 C	β1,**2**; Ser1β1
(7)	α	55.5, CH	4.50, m	C, N, β[g]	
	β1 (*pro-S*)	64.9, CH_2	4.01, dd (11.2, 2.9)	C, N	
	β2 (*pro-R*)		3.94, dd (11.1, 2.6)	C, N, α	
	C═O	174.2, qC			

[a] Numbers in parentheses indicate residue number in atom-labeling scheme for crystal structure in Figure 1. [b] Assigned from ^{13}C and DEPT135 (125 mHz). [c] Assigned from $^1H\rightarrow^{15}N$ HSQC and HMBC (500 or 600 mHz). [d] 500 or 600 mHz. [e] Correlations, optimized for 5, 8 Hz ($^1H\rightarrow^{13}C$) and 4, 8 Hz ($^1H\rightarrow^{15}N$), are from proton(s) stated to carbon or nitrogen at indicated position. Correlations to Acc carbons and nitrogens observed at –6 and 4 °C, respectively. Stronger correlation to one member of a geminal pair indicated in bold. [f] Inter-residual correlations are indicated for both partners and, thus, appear twice in column; intraresidual correlations are indicated once. [g] Weak correlation.

and 71.1 consistent with oxygenated carbons. This left a single quaternary carbon at δ_C 37.5 and 12 methylenes, including three producing signals consistent with oxygenation at δ_C 60.8, 62.8, and 64.9 and two upfield signals at δ_C 13.5 and 12.9, consistent with the deshielding effect of a strained ring.

A dqCOSY spectrum of **1**, featuring four key cross-peaks correlating doublet signals in the amide region with multiplets in the α-methine region, indicated the peptidal nature of the compound. Using these correlations as entry points, the side-chain spin systems of three serine units and a γ-substituted lysine unit (HyLys) were deciphered and assigned. In addition, COSY correlations between an amide proton triplet at δ_H 7.99 and a pair of geminal protons at δ_H 3.73 and 3.06, which, in turn, correlated with another geminal pair at δ_H 2.38 and 2.28, were consistent with the presence of a β-alanine unit. A conspicuous set of signals from a spin system consisting of two pairs of methylene protons attached to adjacent carbons, with upfield resonances between δ_H 0.7 and 1.6 and vicinal coupling constants of ca. 10 and 5.5 Hz for each pair, suggested the presence of a cyclopropyl ring. All remaining cross-peaks in the COSY spectrum could be accounted for by the presence of a γ-hydroxyproline unit.

A combination of ^{13}C and ^{15}N HSQC and HMBC experiments at ambient and reduced temperatures confirmed the presence of the amino acid units deduced from the COSY data. The cyclopropyl group was assigned to a residue of 1-aminocyclopropane-1-carboxylic acid (Acc) on the basis of ^2J HMBC correlations between the broad downfield amide proton singlet and the quaternary α-carbon at δ_C 37.5, and between the upfield β-proton resonances and the α- and β-carbons (Table 1).

HMBC correlations between amide and α-protons and carbonyl carbons established the sequence and cyclic nature of peptide **1** and, in combination with other 2D NMR data, permitted complete

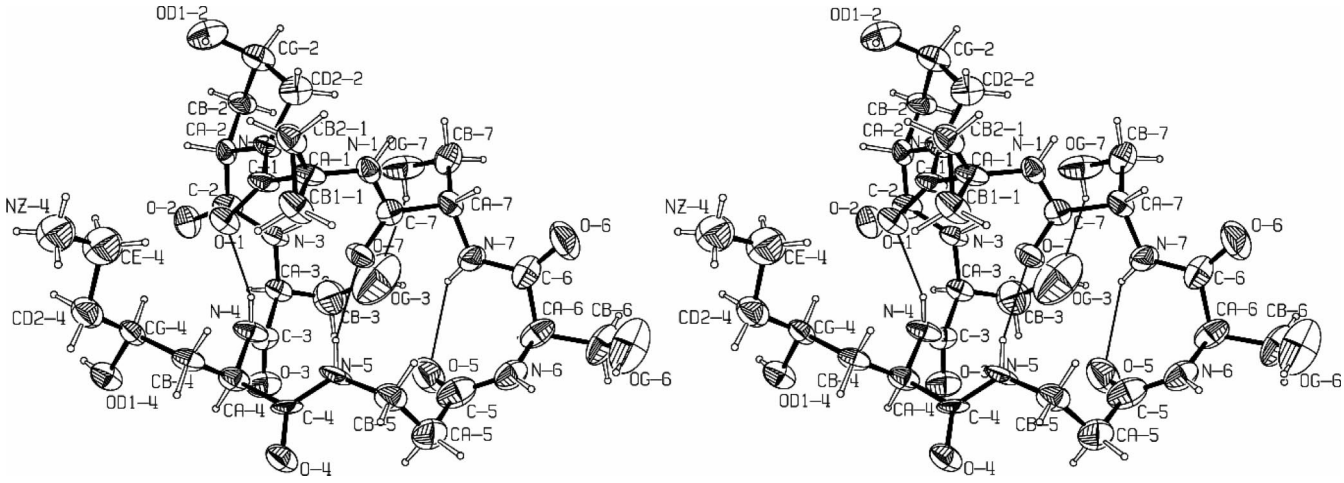

Figure 1. Stereoview of crystal structure of **1**, with 50% probability thermal ellipsoids for non-H atoms. Thin lines are drawn for probable hydrogen bonds. Solvent species are omitted, and only the higher occupancy conformation of the HyLys side chain is shown.

^{1}H, ^{13}C, and ^{15}N assignments (Table 1). The structure of **1** accounts for the seven exchangeable protons not seen in NMR spectra (five hydroxyls and the two HyLys ε-amino protons) and satisfies the 10 degrees of unsaturation indicated by the molecular formula.

Interpretation of NMR spectra was complicated by the presence of a singlet at δ_H 8.53 that integrated as a single proton in ^{1}H spectra of all samples of the compound. We surmised that this proton resonance results from formate, as it correlated to a broad carbon signal at δ_C 169.3 in HSQC spectra. This indicated that **1** purified as a hydroformate salt that formed during the extraction process and survived multiple purification procedures without dissociating. This conclusion was supported by X-ray diffraction data showing that, in a crystal of **1**, a formate ion was present adjacent to the ε-amine group, with a distance of ca. 2.8 Å between the amino nitrogen and an oxygen atom of the formate.

Several confusing features of 1D proton spectra acquired at 25 °C were clarified by spectra taken at reduced temperature. Incorporating presaturation into pulse sequences to suppress the strong CD_3OH solvent signal resulted in a significant reduction in the size of the amide proton peaks from Ser2 and Acc in addition to excessive broadening of the latter, indicating that these amide protons exchange with the OH from CD_3OH. The weakness of the Ser2 amide signal initially suggested that it came from a contaminating analogue (see Supporting Information). The amide proton signal from Acc was not seen at all in spectra taken with solvent suppression at 25 °C and was first revealed as a broad singlet at δ_H 9.50 in spectra taken without solvent signal presaturation. Correlations to this peak in proton-detected 2D spectra were revealed by cooling the probe. This had the effect of slowing solvent exchange and sharpening the peak (and also moving the signal downfield) (Table 1 and Supporting Information). Thus, in a ^{15}N HSQC experiment carried out at 4 °C, a cross-peak was observed between a broad proton singlet at δ_H 9.60 and a ^{15}N resonance at δ_N 125.7, which was then assigned to the Acc amide nitrogen. In a ^{13}C HMBC experiment, cooling the probe to −6 °C was necessary to sharpen the Acc amide proton peak sufficiently to see a cross-peak with the carbonyl carbon of Ser3, which was the final connectivity necessary to confirm the cyclic nature of **1**. Circumventing this problem by the use of nonexchanging solvents was considered at first, but both serinocyclins A and B proved insoluble in chloroform, dichloromethane, and acetone. DMSO was avoided because of the difficulty of recovering material for bioassays and hydrolysis.

Direct chiral amino acid analysis by Cu^{2+} ligand-exchange chromatography indicated the presence of L-Ser, D-Ser, 2*S*,4*R*-Hyp, β-Ala, and Acc. This result was confirmed by HPLC using mass detection of Marfey's derivatives, which also revealed a peak attributable to the HyLys derivative, although determination of its chirality was not possible due to the lack of relevant standards. The peak area ratios for L-Ser:D-Ser (1.5 and 1.6 for the ligand exchange and Marfey's methods, respectively) were consistent with the presence of two L-Ser and one D-Ser residues.

The crystal structure of **1** was determined using X-ray diffraction and standard techniques (Figure 1).[16] This structure, along with mass and NMR spectroscopic data and the results of chiral amino acid analyses, allowed assignment of the complete absolute configuration of all stereocenters in **1**.

ROESY data (Table 1) indicated that the three-dimensional solution structure of serinocyclin A closely resembles that of the crystal structure. Several key transannular ROESY correlations established proximity of protons located on opposite sides of the macrocycle. The HyLys amide proton correlated with both the Acc β1a (δ_H 1.60) and β1b (δ_H 0.73) protons, with the stronger correlation to the former. This was consistent with the crystal structure, which indicated that the HyLys amide is closer to the Acc β1a proton (3.76 Å) than to Acc β1b (4.55 Å). The β-Ala amide proton showed cross-peaks of approximately equal intensity with both Acc β1a and b protons, consistent with the approximately equal interatomic distances between these proton pairs (3.84 and 3.81 Å, respectively). These observations suggested that the Acc β1 protons project toward the middle of the ring. In support of this view, the Acc β2 protons correlated with the Hyp δ2 proton (δ_H 3.73), suggesting that they face away from the interior of the ring. A cross-peak between the Ser1 β1 proton (δ_H 4.22) and the amide protons of HyLys, β-Ala, and Ser3 indicates that the side chain of Ser1 projects centrally, as it does in the crystal structure in which these correlating proton pairs are separated by 4.11, 4.50, and 4.14 Å, respectively.

Interatomic distances from the crystal structure of **1** along with ROESY data supported stereospecific assignments of the diastereotopic protons in the Acc, Hyp, Ser1, Ser2, and Ser3 units (Table 1).

The atom pairs most likely to be involved in hydrogen bonds, based on interatomic distances in the crystal structure, are the Acc carbonyl and the HyLys amide proton (2.12 Å), the β-Ala amide and the Ser3 carbonyl (2.10 Å), and the Ser3 amide and the β-Ala carbonyl (2.29 Å). Hydrogen bonding is also likely between the Ser1 and Ser3 side chains, as the distance between the two hydroxyl oxygens in the crystal is 2.91 Å. The aforementioned proclivity of the amide protons of Acc and Ser2 to undergo solvent exchange suggested that they were not involved in intramolecular hydrogen bonds and probably project out and away from the interior of the ring, and, thus, are more exposed to solvent than the other amides. This view is also supported by the crystal structure and by the strong

Table 2. NMR Spectroscopic Data (CD_3OH) for the Lys Unit of Serinocyclin B (**2**)

unit	position	δ_C[a] (or δ_N[b]), mult	δ_H[c] (J in Hz) [$-\Delta\delta/\Delta T$ ppb/K]	HMBC[d]	NOESY
Lys	NH	120.5, NH	7.53, d (7.8) [1.3]	α, *β*; Ser-1 C	α, *β*, *γ***1**,2, *ε*; *β*-Ala NH
	α	56.0, CH	4.30, ddd (7.6, 7.6, 7.6)	C, N, *β*, *γ*; Ser-1 C	*β*, *γ***1**,2
	β	31.8, CH_2	1.94, m	N, *γ*, *δ*	*β*-Ala NH
	*γ*1	24.3, CH_2	1.61, m	α, *δ*	
	*γ*2		1.46, m	α, *β*, *ε*	
	δ	27.9, CH_2	1.70, m	*β*, *γ*, *ε*, *ε*-N	
	ε	40.8, CH_2	2.90, t (7.1)	*γ*, *δ*, *ε*-N	*β*, *γ***1**,2, *δ*
	ε-NH_3	31.3, NH_3^+		*δ*, *γ*, *ε*-N	
	C=O	174.7, qC			

[a] 125 mHz. [b] Assigned from $^1H\rightarrow^{15}N$ HSQC and HMBC (500 or 600 mHz). [c] 500 mHz [d] Correlations, optimized for 5, 8 Hz ($^1H\rightarrow^{13}C$) and 4, 8 Hz ($^1H\rightarrow^{15}N$), are from proton(s) stated to carbon or nitrogen at indicated position.

negative temperature-dependent changes in chemical shifts of these amide protons (Table 1).[17]

HRESIMS data for **2** were consistent with the molecular formula $C_{27}H_{44}N_8O_{11}$, indicating that **2** differs from **1** by the absence of a single oxygen atom. This suggested the replacement of one of the hydroxyl groups in **1** with a proton. NMR data for the two compounds were essentially identical except for signals associated with the *γ*-hydroxylated lysine unit of **1**. ^{13}C and DEPT135 data showed that the signal at δ_C 66.8 from the substituted *γ*-carbon of this unit in **1** was replaced in **2** by a methylene carbon at δ_C 27.9. Concomitantly, the downfield shifted signal from the proton attached to this carbon atom was replaced by signals at δ_H 1.61 and 1.46 that were readily assigned to a pair of geminal protons. Upfield shifts in proton and carbon signals assigned to the α, *β*, *δ*, and *ε*-positions were consistent with the absence of an electron-withdrawing substituent on the *γ*-carbon (Table 2).

As with **1**, all vicinal couplings could be identified and assigned from the COSY spectrum of **2** (see Supporting Information). The HMBC correlation between the Acc amide proton and the Ser3 carbonyl was not observed for **2** due to the exchange-related attenuation of the Acc amide signal described above. However, all other key interresidual connectivities were observed, and further detailed analyses of 2D NMR data allowed complete ^1H, ^{13}C, and ^{15}N assignments for **2** and supported the conclusion that the position occupied by the *γ*-hydroxylated lysine in **1** is occupied by a lysine unit in **2** (see Supporting Information).

Chiral amino acid analysis revealed that the amino acid composition of **2** is identical to that of **1** except for the replacement of the HyLys unit by D-Lys. This lent further support to the structure of **2** derived from the NMR data. As for **1**, the peak area ratios for L-Ser:D-Ser (1.8 and 1.6 for the ligand exchange and Marfey's methods, respectively) indicated two L-Ser and one D-Ser residues. The position of the D-Ser unit in **2** was deduced by analogy to **1**. Detailed chiral amino acid analysis data for both **1** and **2** are tabulated in the Supporting Information.

Nonproteinogenic amino acids such as the D-Ser, D-Lys, Acc, and *β*-Ala found in the serinocyclins indicate nonribosomal biosynthetic origins. Among NRPs, the only prior reports of an Acc unit are two from *Streptomyces*[18,19] and two from fungi.[20,21] *γ*-Hydroxylysine is likewise scarce among NRPs. It occurs as the (2*S*,4*R*)-isomer in cerexins A and B from *Bacillus cereus*[22] and as the (2*S*,4*S*)-isomer in the cepafungins, products of an undescribed *Pseudomonas* sp.,[23] and in the related glidobactins from *Polyangium brachysporum*.[24] To our knowledge, this is the first report of a *γ*-hydroxylysine unit from a fungal peptide, as well as the first report of the (2*R*,4*S*)-isomer from either fungal or bacterial NRPs. *β*-Hydroxylysine occurs in the victorins produced by the Victoria blight fungus, *Cochliobolus victoriae*.[25]

The extensive hydroxylation and the free lysyl *ε*-amine group, which should be cationic at neutral pH and below, impart considerable hydrophilic character to the serinocyclins. Another noteworthy chemical feature contributing to their polarity is the absence of methyl groups. In a recent review,[26] all but one of more than 40 compounds selected to represent the structural diversity of fungal cyclic peptides contain at least one methyl group, and the single counterexample, trapoxin A, achieves significant lipophilicity from two phenyl groups and a five-carbon methylene chain.[27] Thus, in the context of fungal cyclic peptides, the majority of which are replete with hydrophobic side groups, the serinocyclins are unusual.

Serinocyclin A did not exhibit any detectable antibacterial or antifungal activity at 100 *μ*g/disk in disk diffusion assays in which positive controls tested at 10 *μ*g/disk yielded large (>5 mm) kill zones at 48 h. Likewise, the compound exhibited no detectable self-inhibitory activity at 200 ppm in a conidial germination inhibition assay.

Mosquito larvae exposed to serinocyclin A exhibited a marked inability to swim that can be characterized as a failure to move directionally, either entirely or partially, even though flexion of the abdomen appears to be unaffected. Normal mosquito larvae progress "backward" in a rapid swimming form of locomotion in which they are propelled by quick lashing movements of the abdomen.[28] The EC_{50} for this effect determined by fitting the dose−response curve to the probit model[29] was 59 ppm (see Supporting Information).

Examination of high-speed video of untreated larvae suggests that a mechanism(s) that keeps the head stabilized and relatively fixed in space as the abdomen unflexes enables normal rearward movement. This mechanism does not function properly in treated larvae, so, although the abdomen flexes, the larvae do not move posteriorly as they would normally. This is reflected in the tracks of the tail, center (the first abdominal segment), and the head of a treated insect, which are highly disordered relative to an untreated subject. This departure from the normal is especially evident in the track of the head (see Supporting Information). The kinematics of a slow "gliding" or "browsing" form of locomotion that depends on propulsion by clusters of hairs called mouth brushes has been characterized in detail.[30] The role of the mouth brushes in stabilizing the head during swimming and the possibility that serinocyclin A selectively affects their deployment requires further investigation.

We do not propose that the observed effect of serinocyclin A on swimming in mosquitoes is directly related to the evolved function of these compounds, which remains elusive given the lack of antimicrobial and self-inhibitory activity. Future research should be directed toward determining how these compounds contribute to the fitness of the producing strains.

Experimental Section

General Experimental Procedures. Optical rotations were measured at 25 °C in MeOH on a Perkin-Elmer 241 polarimeter using the sodium lamp (589 nm) with a 100 mm cell. NMR spectra were acquired on Varian Inova 500 and 600 spectrometers using DBG broadband decoupler (500 only) and HCN inverse detection probes. ^{13}C NMR and DEPT spectra were acquired at 125 MHz. $^1H\rightarrow^{13}C$ HSQC and HMBC spectra were acquired at 500 or 600 MHz (^1H-dimension), and experiments were optimized for $^1J_{CH} = 150$, 190 Hz and $^nJ_{CH} = 8.0$, 5.0, respectively. $^1H\rightarrow^{15}N$ HSQC and HMBC spectra were acquired at 500 or 600 MHz (^1H-dimension), and experiments were optimized for $^1J_{CH} = 95$ Hz and $^nJ_{CH} = 4.0$, 8.0, respectively. ROESY and NOESY

spectra were acquired with mixing times of 120 and 200 ms, respectively. Amide thermal chemical shift coefficients ($-\Delta\delta/\Delta T$, ppb/K) were derived from curves for values in the range from −6 to 40 °C. All NMR data were recorded for samples dissolved in CD_3OH. ^1H chemical shifts were referenced to the center of the residual CHD_2OH quintuplet at δ_H 3.31. ^{13}C chemical shifts were referenced to the center of the $^{13}CD_3OH$ septuplet at δ_C 49.15. ^{15}N chemical shifts were referenced externally to the ^{15}N signal of benzamide at 104.5 ppm downfield of liquid NH_3. Solvent signal suppression in dqCOSY and HMBC spectra was accomplished by modifying the pulse sequence with a presaturation delay of 1 s. Low-resolution ESI mass spectra were acquired in positive ion mode by infusion of methanolic solutions at 5 μL/min via a syringe pump (Harvard Apparatus) into a Micromass ZMD 4000 spectrometer (capillary and sample cone voltages of 3.5 kV and 60 V, respectively). High-resolution ESI mass spectra were similarly acquired in positive ion mode using a Micromass Autospec spectrometer.

Fungal Material and Culture Conditions. Cryogenically preserved mycelial material of *M. anisopliae* var. *anisopliae* ARSEF #2575 or *M. anisopliae* var. *acridum* strain GMD was used to produce conidial stocks, which were stored at −80 °C in 10% glycerol. These stocks were used to inoculate starter cultures on 9 cm plates of 25% ("quarter-strength") Sabouraud's dextrose agar plus yeast extract (SDAY) prepared as described previously.[10] After 7–10 days, conidia from these plates were spread with a sterile cotton swab on 9 cm plates of barley agar (15 g each of barley flour and agar per liter of H_2O) for bulk production of conidia. After 7–10 days, conidia were harvested by brushing them onto a 75 μm mesh stainless steel sieve to remove mycelial particles and collecting them in a stainless steel pan. Conidia were held in a desiccator under vacuum overnight to remove traces of water and then transferred into tared 50 mL polypropylene centrifuge tubes and weighed. Sixty plates yielded ca. 3 g of conidia.

Extraction and Isolation. A sequential extraction protocol was developed to optimize the yield of **1** and **2**. Conidia were suspended in hexane (30 mL/0.5 g conidia), vortexed for 30 s, and then centrifuged at 840*g* for 5 min to pellet conidia. The supernatant was concentrated in vacuo, and the conidia were resuspended in MeOH by vortex mixing (30 mL/0.5 g conidia). Conidia were then extracted by sonication in MeOH for 30 min, after which they were centrifuged as above, the supernatant was concentrated, and the pellet was resuspended in 30 mL of MeOH/1% formic acid. This fraction was treated identically to the MeOH fraction.

The concentrated supernatants from the MeOH/1% formic acid extraction of 3 g of conidia were combined and dried in vacuo to yield 450 mg of a brown oil. This was then dissolved in MeOH and chromatographed on Sephadex LH-20 (25 × 900 mm) eluted with MeOH at 4.5 mL/min. One-hundred 2 min fractions were collected and recombined on the basis of ESIMS analysis. Fractions 27–29, which gave the strongest signals from the 672 Da compound, were combined to yield a brown oil (83.3 mg), which was further fractionated by repeated semipreparative reversed-phase HPLC (Phenomenex Synergi Fusion RP; 10 × 250 mm; 4 μm; 80 Å) eluting at 4 mL/min using 100% H_2O for 8 min, followed by a linear gradient from 0 to 100% MeCN in 10 min, and holding at 100% MeCN for 2 min with UV detection (λ = 215 nm) to furnish **1** (27.3 mg) and **2** (5 mg) of >99% purity (t_R = 9.2 and 12.4 min, respectively).

Serinocyclin A (1): colorless plates (MeOH); $[\alpha]^{24}_D$ +120.6 (*c* 0.65, MeOH); ^1H, ^{13}C NMR, HMBC, ROESY data, see Table 1; HRESIMS obsd *m/z* 673.3159 $[M + H]^+$, calcd for $C_{27}H_{44}N_8O_{12}$ + H 673.3157; HPLC t_R = 9.2 min.

Serinocyclin B (2): colorless oil; $[\alpha]^{24}_D$ +118.1 (*c* 0.52, MeOH); ^1H, ^{13}C NMR, HMBC, NOESY data, see Table 2 and the Supporting Information; HRESIMS obsd *m/z* 657.3234 $[M + H]^+$, calcd for $C_{27}H_{44}N_8O_{11}$ + H 657.3208; HPLC t_R = 12.4 min.

Direct Chiral Amino Acid Analysis. Samples of **1** and **2** (1 mg) were hydrolyzed in 1 mL of 6 N HCl at 110 °C for 20 h and then dried in vacuo. The hydrolysates were dissolved in 1 mL of H_2O, and a 10 μL aliquot was analyzed directly by HPLC using a ligand-exchange column (Phenomenex Chirex (D)-penicillamine; 4.6 × 250 mm; 5 μm) eluted at 1 mL/min with 5% 2-propanol in 2 mM aqueous $CuSO_4$ with detection by UV absorption at 254 nm. Amino acid constituents of the hydrolysates were identified by comparing peak retention times with those of the following standards: D- and L-Ser, D- and L-Lys HCl, (2*S*,4*R*)-Hyp, (2*R*,4*S*)-Hyp HCl, (2*S*,4*S*)-Hyp, (2*R*,4*R*)-Hyp, as well as the achiral β-Ala and Acc. Standards for HyLys isomers were not commercially available. Amino acid identifications were confirmed by cochromatography experiments using appropriate standard mixtures and by HPLC-ESIMS experiments. All amino acid standards were purchased from Sigma except for 2(*R*,4*S*)-Hyp, which was purchased from Astatech, Inc. (Bristol, PA).

Chiral Amino Acid Analysis Using Marfey's Method. The ligand-exchange method did not resolve β-Ala from L-Lys and did not afford baseline separation of (2*S*,4*R*)-Hyp, L-Ser, and D-Ser. Therefore, hydrolysates were analyzed by Marfey's method using both UV and ESIMS detection[31,32] to verify the identity of amino acid derivatives. Dried hydrolysates were dissolved in water (ca. 5 mg/mL). To a 50 μL aliquot of the hydrolysate solutions were added 1% (w/v) 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent (Pierce)) in acetone (20 μL) and 1 M $NaHCO_3$ (20 μL). The mixture was held at 40 °C for 1 h, after which the reaction was quenched with 1 M HCl (20 μL). Standard amino acids (see above) were derivatized in a similar manner. Samples were dissolved in mobile phase and analyzed by reversed-phase HPLC using isocratic mixtures of (A) MeOH (800)/MeCN (200)/5 M ammonium acetate (3) and (B) 15 mM ammonium acetate. Cochromatography experiments employed 30% A:70% B, Phenomenex Luna C18; 4.6 × 100 mm; 5 μm, 100 Å; flow rate 1.0 mL/min, detection by UV absorbance at 347 nm. HPLC-ESIMS experiments employed 35% A, 65% B, Phenomenex Prodigy ODS3; 2.0 × 150 mm; 5 μm; 100 Å; flow rate 0.25 mL/min, diode array UV (Waters 996 210-500 nm in 1 s) and ESIMS detection (Micromass ZMD-4000 spectrometer, scanning *m/z* 180–700 in 1.5 s in negative ion mode, capillary and cone voltages 3.25 kV and 40 V, respectively, source block and desolvation gas temperatures 150 and 250 °C, respectively).

X-ray Crystallography of 1. A single crystal of **1**, measuring 0.06 × 0.06 × 0.003 mm, was obtained by slow evaporation from MeOH. It was harvested on a MiTeGen MicroMount,[33] using a small amount of mineral oil for adhesion (type 300 immersion oil, Hampton Research HR3-611). Data were collected at 100 K with a wavelength of 0.9179 Å at the Cornell High Energy Synchrotron Source F1 beamline. A microcrystal setup, including capillary focusing (beam diameter 0.018 mm) and a helium enclosure around the crystal, was used. Sixty 5-deg φ-wedges (6 s exposure time) were recorded with an ADSC Q-270 CCD detector to a limiting resolution of 1.0 Å ($2\theta_{max}$ = 54.6°). Data were integrated with MOSFLM[34] and scaled with SCALA.[35] Observations (n = 5536) were processed and merged to give 1670 unique reflections (R(merge) = 5.8%, completeness 89%), of which 1600 (1528 with $I/\sigma(I) > 2$) were used for structure solution and refinement and 70 were reserved for calculating R(free). A TAILS correction (range 1.003 to 1.237) and an absorption correction (range 0.961 to 1.045) using spherical harmonics were applied in SCALA.[36] The structure was solved by direct methods and refined using full-matrix least-squares on F^2 and difference Fourier maps.[37,38] Suitable restraints were applied to bond lengths and angles. In addition to molecule **1**, three water molecules and a formate ion were located in the asymmetric unit. The formate and the end of the adjacent HyLys side chain were present in two alternate conformations in approximately a 60/40 ratio. Hydrogen atoms were added in stereochemically reasonable positions using a "riding" model and not refined. Anisotropic thermal parameters were refined for all non-hydrogen atoms except for those with alternate conformations (for which isotropic U's were refined). At convergence, using 94 restraints, R1 = 0.0889, R(free) = 0.1371, and GooF = 1.342 for 475 parameters.

Mosquito Assay. Eggs of *Aedes aegypti* (Rockefeller strain) were hatched under vacuum for 30 min and maintained at 25 °C in ca. 200 mL of tap water supplemented with 1 mL of a 0.5% w/v liver powder suspension. At 24 h posthatch, 7–11 larvae were transferred in 0.9 mL of food/water mixture to wells (3.4 mL capacity) of a 24-well plate. Treatments were added in 100 μL of distilled water. Effects were observed and scored at 24 h after treatment.

Microbiological Assays. Serinocyclin A was tested against bacterial target strains (*Escherichia coli* and *Bacillus cereus*) grown on nutrient agar plates (9 cm) and against the fungal strains *Colletotrichum acutatum*, *Botrytis cinerea*, and *Beauveria bassiana* (ARSEF #8354), grown on potato dextrose agar plates, and *Saccharomyces cerevisiae* grown on 1% yeast extract, 2% peptone, 1.5% agar, supplemented with either 2% dextrose or 2% glycerol. Plates were spread with 0.6 mL aliquots of a liquid bacterial culture grown for 24 h in nutrient broth, 0.6 mL of a fungal conidial suspension (ca. 1 × 10^6 conidia/mL H_2O), or 0.5 mL of a yeast suspension in H_2O adjusted to OD_{600} = 0.5.

Inoculated plates were allowed to dry for 1 h. Test samples were applied to 7 mm filter paper disks in 10 μL of MeOH. Disks were air-dried for 1 h before being placed on assay plates. Cultures were monitored for zones of inhibition every 24 h for 4 days. Tetracycline and filipin were used at 10 μg/disk as positive controls for the bacterial and fungal targets, respectively. MeOH and formic acid were used at 10 μL/disk as negative controls.

To test serinocyclin A for self-inhibition of germination, a conidial suspension of GMD was prepared at a concentration of 7×10^5 conidia/mL in a minimal germination medium (2 g of glucose/2 g of yeast extract/1 L of H_2O).[39] Conidia were distributed to flat-bottomed wells of a 96-well plate in 90 μL of medium. Serinocyclin A solutions (0.5, 1.0, and 2.0 mg/mL of H_2O) were delivered to the wells in 10 μL aliquots to afford final concentrations of 50, 100, and 200 ppm. The controls consisted of deionized water (18 Ω) and formic acid at a final concentration of 10 ppm. Plates were incubated at 25 °C, and germination was evaluated using an inverted compound microscope at 16 h after treatment.

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Supporting Information Available: ^1H, ^{13}C, and COSY spectra of **1** and **2**; X-ray data for **1**, chiral amino acid analysis data for **1** and **2**, complete NMR assignments, HMBC, and NOESY data for **2**, and bioassay data for **1** are available free of charge via the Internet at http://pubs.acs.org.

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