Discovery, Synthesis, and Insecticidal Activity of Cycloaspeptide E

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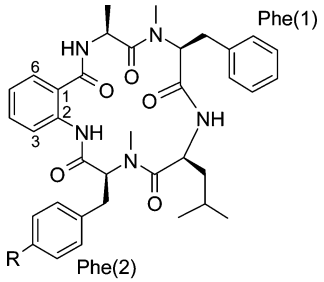
Several *Penicillia* and one *Tricothecium* strain produced a new, insecticidally active member of the cycloaspeptide family, with the proposed name cycloaspeptide E (**1**). The structure, which was determined on the basis of spectroscopic (NMR, UV, MS) data and Marfey amino acid analysis, was the tyrosine desoxy version of cycloaspeptide A (**2**). Two synthetic routes to compound **1** were developed: one a partial synthesis from **2** and the other a total synthesis from methyl alaninate hydrochloride. Cycloaspeptide E, the first member of this series not to contain a tyrosine moiety, is also the first to be reported with insecticidal activity.

Fungi continue to be an exciting source of novel active molecules that add to chemical diversity and contribute screening inputs to both pharmaceutical and agrochemical lead generation programs. As part of our ongoing search for natural products with potential value as agrochemicals, a set of fungi was collected over several years from leaf litter, wood, and twigs. Initial screening of small fermentations of these fungi revealed several strains (Table S1; Supporting Information) that were insecticidally active. LC-MS-bioassay profiling showed the presence of the same unidentified, low-abundance insecticidal compound in each strain. These observations prompted us to perform bioassay-directed isolation of the active molecules from large-scale fermentations of several of these strains. Three molecules were isolated and identified as a result: penitrem A,[1] a known insecticidal metabolite, was identified from one strain (DA051320); a small amount of a novel, bioactive cyclic peptide (**1**) related to cycloaspeptide A (**2**) and a larger amount (approximately 60× the level of **1**) of cycloaspeptide A[2,3] itself were present in all strains examined. Interestingly, **2** was inactive, although it was helpful in the structure elucidation of **1** and in enhancing our understanding of the structure−activity relationship for this series. In order to further understand the potential for this class of peptides as insecticidal agents, the synthesis of cycloaspeptides was investigated, and two successful routes to these compounds are reported here.



(**1**) R = H

(**2**) R = OH

(**3**) R = OTf

Both **1** and **2** showed λ_{max} = 256 and 307 nm in their diode array UV spectra during HPLC. Further, ESIMS determined the molecular weight of **1** to be 625, i.e., 16 units less than for **2**, suggesting **1** to be a desoxy analogue of **2**. High-resolution ESIMS supported this proposal, with an experimentally determined accurate molecular weight for **1** of 625.3286, in good agreement with the calculated weight for the desoxy analogue of **2** [$C_{36}H_{43}N_5O_6$ = 625.3264]. ^{1}H NMR revealed that the tyrosine residue present in **2** had been replaced with a phenylalanine group in compound **1**; otherwise the amino acid constituents were the same (Table 1). Analysis of an HMBC spectrum indicated that the amino acids in **1** were connected in the same order as for **2**. Finally, a small amount of **1** was hydrolyzed, derivatized using Marfey's reagent,[4] and analyzed by LC-MS. This confirmed the presence of *N*-methylphen-

Table 1. NMR Data for Cycloaspeptide E (**1**) at 600 MHz (150 MHz for ^{13}C) in $CDCl_3$

residue	position	δ_C	δ_H
Ala	NH		6.60 (1H, d, *J* = 7.0 Hz)
	Cα	44.4	4.41 (1H, dq, *J* = 7.0, 6.6 Hz)
	C*β*	16.8	0.43 (1H, d, *J* = 6.6 Hz)
	CO	173.9	
Phe(1)	N-Me	30.4	2.89 (3H, s)
	Cα	63.7	5.21 (1H, dd, *J* = 11.8, 3.0 Hz)
	C*β*	34.4	3.49 (1H, dd, *J* = 14.3, 2.6 Hz)
			3.05 (1H, dd, *J* = 14.9, 12.3 Hz)
	C1	139.1	
	C2−5	129	7.36−7.28 (4H, m)
	C6	127	7.35 (1H, m)
	CO	168.3	
Leu	NH		7.14 (1H, d, *J* = 8.7 Hz)
	Cα	48.8	4.72 (1H, ddd, *J* = 8.5, 8.5, 5.6)
	C*β*	41.7	1.88 (1H, m)
			1.38 (1H, m)
	C*γ*	25.2	1.71 (1H, m)
	C*δ*	23.7	1.02 (3H, d, *J* = 6.7 Hz)
	C*ϵ*	22.4	1.01 (3H, d, *J* = 6.7 Hz)
	CO	170.2	
Phe(2)	N-Me	39.5	2.70 (3H, s)
	Cα	70.0	3.87 (1H, dd, *J* = 10.8, 3.7 Hz)
	C*β*	33.4	3.62 (1H, dd, *J* = 13.8, 4.1 Hz)
			3.47 (1H, dd, *J* = 14.3, 11.3 Hz)
	C1	138.1	
	C2−5	129	7.36−7.28 (4H, m)
	C6	127	7.35 (1H, m)
	CO	171.0	
ABA	NH		12.03 (1H, s)
	C1	115.4	
	C2	142.0	
	C3	121.4	8.95 (1H, d, *J* = 8.7 Hz)
	C4	134.7	7.51 (1H, dd, *J* = 8.7, 8.2 Hz)
	C5	122.7	7.02 (H, dd, *J* = 8.2, 8.2 Hz)
	C6	127.2	7.45 (1H, d, *J* = 8.2 Hz)
	CO	170.0	

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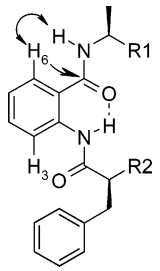


Figure 1. 2D representation of the structure of the anthranilic acid portion of the cycloaspeptides. The single-headed arrow indicates a key HMBC correlation, whereas the double-headed arrow denotes a key NOE signal determining the assignments of H-6 and H-3. Note the proximity of H-3 to the Phe(2) carbonyl, causing a large downfield shift for H-3.

ylalanine, alanine, and leucine as the constituent amino acids. This analysis also showed that each amino acid was present in the L-configuration, as had been observed for previous members of this series.[2,3,5] Thus the structure of **1** was confirmed to be the desoxy analogue of **2**. Large-scale fermentations of strains DA087002, DA087027, DA087041, and DA087044 gave similar relative production levels of **1** and **2** without production of penitrem A.

One noteworthy feature of the NMR spectra of the cycloaspeptides is the far downfield chemical shift of the anthranilate H-3 in all solvents. In earlier assignments of the structure[2,5] the doublet signal at δ 8.5 was assigned to H-6. However in this work, this peak did not show a cross-peak to the anthranilate carbonyl atom in any HMBC experiments; rather, the doublet at δ 7.45 did. Additionally, in gsNOE experiments, irradiation of Ala-NH gave an enhancement to this doublet, assigning it to H-6, and H-3 to the signal at δ 8.95. The reason for this low-field shift for H-3 may be attributed to a strong hydrogen bond between the anthranilic acid NH and carbonyl. With a small cyclic peptide, this forces the carbonyl of the phenylalanine (or tyrosine in cycloaspeptides A−D) toward the plane of the anthranilic acid phenyl ring, thus strongly deshielding H-3 and also bringing Ala-NH close to H-6 (Figure 1). These observations were consistent between different solvents (DMSO, $CDCl_3$, and MeOD).

The observation of lepidopteran activity for compound **1** was novel within this series of natural products, and the activity differential between **1** and its OH-analogue **2** prompted us to further investigate this series. While milligram amounts of **1** and gram amounts of **2** were subsequently obtained by scale-up fermentation and isolation, this was not considered a sustainable source to provide starting material for synthesis, especially if analogues were needed. Since structure **1** looked synthetically approachable either by total synthesis or via partial synthesis from naturally occurring **2**, we decided to investigate both routes.

Partial synthesis of **1** from naturally occurring **2** was accomplished via reduction of the triflate (**3**). While standard methods of aryl triflate reductions either did not work or gave significant hydrolysis, the method of Kotsuki[6] using triethylsilane as the hydrogen source was effective. Milligram-scale formation of the triflate followed by reduction gave 73% purified (HPLC) yield of **1**. Our total synthesis route was modeled on the published route[7] for the related avellanins, using the Fmoc protocol. In the synthesis of the avellanins, a linear peptide was generated starting from methyl-2-aminobenzoate, with macrocyclization being accomplished in 29% yield using diphenyl phosphoramidate (DPPA). It rapidly became apparent that synthesis of the cycloaspeptides could not be similarly accomplished. Thus, deprotection of Fmoc or BOC moieties using standard conditions from a suitable tri- or tetrapeptide precursor to **1** resulted in significant hydrolysis of the benzoate moiety, decreasing yields substantially. Success was accomplished, however, by starting with alanine already linked to the amino benzoate (**4**) and protected as the methyl ester, which could be assembled to the pentapeptide (**5**) in good overall yield with no aminobenzoate cleavage (Scheme 1). Macrocyclization of **5** with DPPA yielded **1** in low yield (10−15%), accompanied by formation of a major byproduct. Evidence from NMR suggested that this byproduct was the acylated aminal (**6**) resulting from azide attack on the phosphoryl-activated alanine carboxyl group, followed by Curtius-type rearrangement (Scheme 2). However, this product was not exhaustively characterized. This side reaction was circumvented by use of the cyclic anhydride of propane-1-phosphonic acid, which yielded **1** in a more respectable 67% yield from **5**.

In the present work, several *Penicillia* and one *Tricothecium* strain were shown to produce the insecticidal pentapeptide cycloaspeptide E (**1**),[8] which was isolated chromatographically guided by its BAW activity. Compound **1** is an analogue of the known cycloaspeptide A, which was also produced by these fungi at much (60×) higher levels, but shown to be insecticidally inactive. Previously, members of this structural class were reported to be produced by *Penicillium algidum*,[2] *P. jamesonlandense*,[9] *P. ribeum*,[9] *P. soppii*,[9] and *P. lanosum*[9] (cycloaspeptide A), *P. ribeum*[5] (cycloaspeptide D), and *Aspergillus* sp. NE-45[2] (cycloaspeptides A, B, and C).

Cycloaspeptide E (**1**) was moderately active against beet armyworm following ingestion from artificial diet, showing an MIC of approximately 10 ppm, whereas, under the same conditions, cycloaspeptide A (**2**) was inactive, with MIC > 500 ppm (Table S2; Supporting Information). By comparison, spinosyn A, the major factor in the commercial product Spinosad, had MIC = 0.02 ppm under these conditions. Cycloaspeptide E showed activity following topical treatment directly to both beet armyworm and cabbage looper, as well as following consumption of treated leaves. Again, under the same conditions, **2** was essentially inactive. Finally, following injection into beet armyworms, test conditions that remove intrinsic barriers to activity (gut hydrolysis, cuticle penetration, etc.), **1** was active, whereas **2** showed only, at best, fleeting initial activity. A characteristic symptomology of larvae injected with **1** was full-body tremors, consistent with a neurotoxic mode of action.

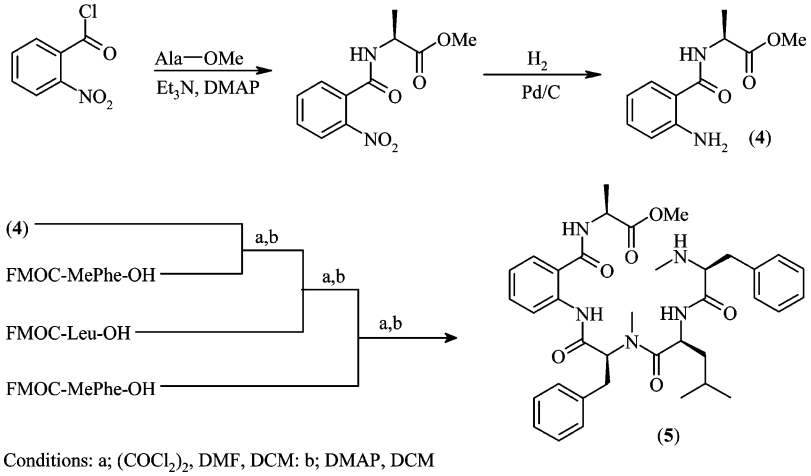
Compound **1** is the first member of the cycloaspeptide series not containing a tyrosine residue, as well as being the first-reported insecticidal example. The total synthesis reported here is the first such route reported for this class. The discovery of compound **1** and its insecticidal activity represents another example of the contribution fungi can play in the generation of novel agrochemical leads via studies of natural products.

Experimental Section

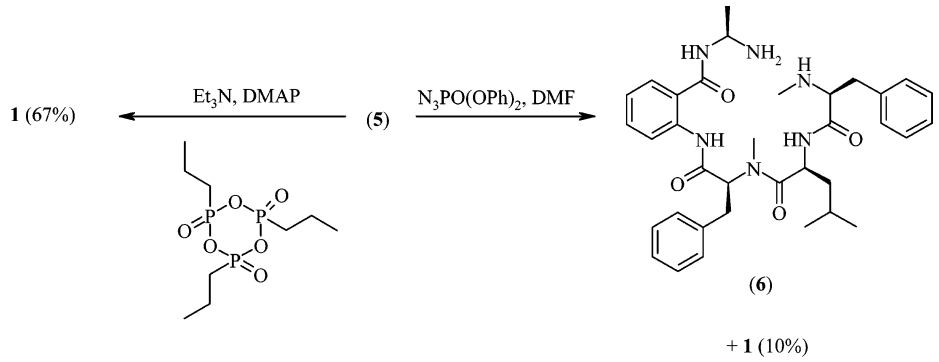
General Experimental Procedures. All NMR spectra were recorded on a Bruker DRX600 spectrometer operating at 600.13 MHz (^{1}H) and 150.62 MHz (^{13}C). HPLC separations were achieved using a Hewlett-Packard 1100 LC system. LC-MS was performed on a Micromass Platform single-quadrupole mass spectrometer in both positive electrospray (+ESI) and negative electrospray (−ESI) modes. Accurate LC-MS and LC-MS/MS experiments were conducted on a Micromass hybrid quadrupole time-of-flight (Q-Tof) instrument.

General Synthetic Procedures. A: Peptide Coupling. Oxalyl chloride (5 equiv) was added in one portion to a stirred solution of Fmoc-protected amino acid (1 equiv) in DCM. DMF (1 drop) was then added and the solution stirred under nitrogen for 1 h. The solvent and excess oxalyl chloride were then removed at reduced pressure. The residue was redissolved in DCM, and this solvent was then removed, affording the crude acid chloride. This crude acid chloride was then dissolved in DCM, and this solution was added in one portion to a well-stirred solution of the deprotected peptide (1 equiv) in DCM. To this solution saturated aqueous $NaHCO_3$ solution was immediately added, and this mixture was well stirred for 1 h. The reaction mixture was then diluted with DCM and H_2O. The aqueous phase was separated and the organic phase washed successively with saturated $NaHCO_3$, 1 N HCl, saturated $NaHCO_3$, and brine, then dried ($MgSO_4$). Concentration left a crude coupled product, which was purified by flash

Scheme 1. Synthesis of Acyclic Precursor **5**



Scheme 2. Cyclization of **5** to Synthetic **1**



chromatography over 130 mL of silica eluted with 5−9% acetone in DCM to afford pure coupled Fmoc-protected peptide.

B: Deprotection of Fmoc Group. To a solution of protected peptide (1 equiv.) in DCM was added 4-(aminomethyl)piperidine (excess) in one portion at ambient temperature. The mixture was then stirred under nitrogen for 1 h. After dilution with more DCM the organic phase was washed successively with water, pH 5.4−5.5 buffer and brine and then dried (Na_2SO_4). Concentration left deprotected peptide which was usually pure enough to be used without further purification.

Fungal Collection and Fermentation Conditions. Fungal strains examined in this study are listed in Table S1 (Supporting Information). These strains are maintained in the Mycosynthetix fungal repository in Durham, NC. Compounds **1** and **2** were isolated from fermentation of strains DA051320, DA087002, DA087027, DA087041, and DA087044, and their structures were determined spectroscopically (NMR, MS, UV). Compounds **1** and **2** were detected in fermentation broths from the remaining strains by LC-MS analysis only. Strain DA051320 did not form aerial mycelia or conidiophores on any media tested; therefore, morphological identification could not be completed. A partial DNA sequence of the large subunit ribosomal RNA gene (28S) was determined for strain DA05132 at Midi Labs (Newark, DE). The DNA sequence was compared to the GenBank database using the BLASTN algorithm (http//www.ncbi.nlm.nih.gov/BLAST/). The strongest BLAST match for DA058132 was *Trichothecium roseum* (U69891). The four remaining strains from which cycloaspeptides were isolated all produced abundant aerial mycelium on a variety of media with a dark green pigment and conidiophores characteristic of *Penicillium* spp. A partial DNA sequence of the small subunit ribosomal RNA gene (18S) was determined[10] for strains DA087041 and DA087044. The strongest BLAST matches for each of these two strains were to various *Penicillium* species. The partial rRNA gene sequences were deposited in GenBank under the following accession numbers: *Tricothecium* sp. DA051320 (DQ497012), *Penicillium* sp. DA087041 (DQ443729), and *Penicillium* sp. DA087044 (DQ443730).

Fungi were cultured on a small scale in multiple media for initial screening purposes. The following protocol was used to prepare larger amounts of broth for scale-up metabolite isolation. A small agar plug, containing fungus from a malt agar slant, was transferred into 7 mL of YESD broth [soy peptone (Sigma P-1265; 2% w/v), dextrose (Sigma G-8270; 2%), yeast extract (Becton Dickinson 4311929; 1%)] in a 50 mL centrifuge tube. This was incubated for 7 days at 22 °C on an orbital shaker at 200 rpm with a 1 in. throw. For 2 L or larger fermentations, the contents of the 7 mL starter culture above were transferred to a 250 mL flask containing YESD medium (75 mL). This was incubated a further 7 days at 22 °C. An aliquot (3 mL) from this secondary culture was used to inoculate either a Nunc bioassay dish containing solid medium (500 mL) or a 2 L Fernbach flask containing liquid medium, and incubating at 22 °C for 11 days. The media[11] used for these scale-up fermentations (Table S1) were as follows: (A) mannitol (0.5%), soy grits (0.2%); (B) glucose (1%), yeast extract (0.1%), casein (0.2%), agar (1.8%); (C) oatmeal agar; (D) Czapek agar; (E) mycological agar, low pH; (F) mannitol (0.5%), soy grits (0.2%), agar (1.8%); (G) potato dextrose agar.

To prepare the completed fermentations for extraction, the plates were frozen at −75 °C for 24 h, then freeze-dried until completely dry. After freeze drying, the dried residue was transferred to a 5 L graduated beaker. One liter of methanol (MeOH) was added to the container and allowed to stand for approximately 10 h. The extract was filtered through 25 cm, grade 417, qualitative filter paper into an Erlenmeyer graduated flask. After filtering, the residue plus paper was rinsed with MeOH (500 mL) and the two MeOH phases were combined, then dried under vacuum.

Insect Screening. Lepidopteran Diet Assay. Diet was dispensed into the wells of a 96-well microtiter plate (100 μL/well) and allowed to cool and dry. Test samples were dried in the wells of a second 96-well plate and then dissolved in acetone−water (50:50; 50 μL) with sonication. The test sample solutions were transferred to the plate containing insect diet and allowed to dry on the surface of the diet. Test compounds were placed thus on the surface of the diet at a series of rates such that their final concentrations (expressed in ppm of test compound per total weight of diet per well) were 0.98−500 ppm, in 2-fold steps. Each well was then infested with 8−10 beet armyworm (BAW) eggs. Test plates were covered with a layer of sterile cotton and then the plate lid. The effects of the test compounds on the

development of the insects were evaluated after a 6-day incubation period at 28 °C. The insecticidal potency of the test compounds was reported as the minimum concentration of compound (MIC, in ppm) required to inhibit insect growth compared with an untreated control.

Lepidopteran Topical Assay. The compounds were dissolved in acetone at 5 mg/mL. One-microliter treatments of the solutions of each compound were applied along the dorsa of each of six third-instar BAW and cabbage looper (CL) (*Trichoplusia ni*) using a microapplicator, resulting in a treatment rate of 5 μg/larva. Solvent-only and untreated insects were prepared as controls. Following topical application, insects were placed individually into the wells of six-well tissue culture plates. Each well also contained a 1 cm^3 portion of artificial lepidopteran diet. Insects were held under ambient laboratory conditions and were observed for symptoms of intoxication and mortality at 24 and 120 h.

Lepidopteran Limited Ingestion Exposure Assay. To measure the impact of limited ingestion exposure, 0.25 cm^2 leaf disks were treated with 1 μL applications of a 5 mg/mL acetone solution of the test materials. Six cabbage disks (for CL) and six cotton disks (for BAW) were treated and then permitted to dry. Solvent control and untreated disks were also prepared. Disks were then offered to six of each species of lepidopteran for a 24 h period. Larvae and leaf disks were held individually in the wells of six-well tissue culture plates, under ambient laboratory conditions. After 24 h, the larvae were offered a 1 cm^3 portion of artificial lepidopteran diet, and mortality rate, symptoms of intoxication, and amount of the leaf disks consumed were noted.

BAW Injection Assay. The compounds were dissolved in acetone at a concentration of 20 mg/mL. To assess the intrinsic activity of the cycloaspeptides, six fourth-instar BAW were each injected with a 0.5 μL dose of test solution (equivalent to 10 μg per insect). Solvent-only and untreated insects were prepared as controls. After treatment, insects were placed individually into the wells of a six-well tissue-culture plate. Each well contained a 1 cm^3 portion of artificial lepidopteran diet. Treated insects were held under ambient laboratory conditions and were observed for symptoms of intoxication at 1, 24, and 48 h, and for mortality at 48 h.

Metabolite Isolation. The dried MeOH extract from 3 L of DA051320 broth (1.9 g) was dissolved in water−dichloromethane (1:1; 400 mL), shaken to partition, and separated. The aqueous phase was extracted with two further aliquots (2 × 200 mL) of DCM and the organic phase dried on a rotary evaporator. The dried sample was dissolved in MeOH (1 mL), and the components were separated by semipreparative HPLC using a Hypersil-C_8-BDS column (250 × 10 mm; 8 μm) with gradient elution from 0% to 100% acetonitrile in 10 mM NH_4OAc. This yielded **2** (3.4 mg) and a mixed fraction of **1** and penitrem A. This mixed fraction was dissolved in MeOH (500 μL) and further separated by HPLC on a Hypersil-C_8-BDS column (250 × 4.6 mm; 5 μm) eluting isocratically with 10 mM NH_4OAc−acetonitrile (1:1) to yield **1** and penitrem A. On the basis of analytical HPLC peak area-matching using **2** from above as a response standard, the amount of **1** isolated was estimated to be approximately 50 μg. NMR data are summarized in Table 1. Fermentations of strains DA087002, DA087027, DA087041, and DA087044 were performed on a scale of tens of liters and worked up using the same methodology to provide larger quantities of **1** and **2**.

Marfey Analysis. Compound **1** was dissolved in MeOH (2 mg/mL). An aliquot (50 μL; approximately 0.15 μmol) was placed in a 1.8 mL amber glass HPLC autosampler vial and dried (Speedvac), and the vial was flushed with nitrogen for 1 min. After adding 6 N HCl (200 μL) and capping, the vial was heated at 110 °C for 15 h. After cooling to room temperature, the sample was dried under vacuum. The residue was dissolved in 1 M $NaHCO_3$ (500 μL) and divided into two equal aliquots. Each aliquot was treated with Marfey reagent (120 μL of 10 mM acetone solution) and allowed to react at 40 °C for 90 min. After cooling, the samples were quenched with 2 N HCl (150 μL), with gentle agitation, then allowed to sit for 5 min before being dried under vacuum. The solid residues were dissolved in 50% aqueous DMSO with sonication for 10 min, then analyzed by LC-MS.

Preparation of Triflate (3) from Natural 2. To a stirred solution of **2** (12.5 mg) in $CHCl_3$ (2 mL) at room temperature was added 2 drops of Et_3N (excess) followed by 2 drops of triflic anhydride (excess). The reaction was stirred for 10 min and monitored by HPLC (Kromasil-C_{18} 4.6 × 150 mm column, linear gradient from 50% to 100% MeCN in H_2O−TFA, 99.95:0.05; pH 2) over 5 min, then hold at 100% MeCN, 1.2 mL/min flow rate; t_R starting material = 3.5 min, t_R product = 5.52 min). EtOH (1 mL) was added when no starting material remained, and the mixture was concentrated to a light brown oil in vacuo. The triflate was found to be stable to both normal and reversed-phase chromatography, aqueous bicarbonate, and dilute aqueous HCl. The crude product was used in the next step without further purification: MS (−ES) *m*/*z* for $C_{37}H_{42}N_5O_8SF_3$ = 772.3 [M − 1]$^-$, 132.9 (base peak); ^{1}H NMR ($CDCl_3$) 0.43 (d, J = 7 Hz), 1.00 (d, J = 7 Hz), 1.01 (d, J = 7 Hz), 1.41−1.35 (m), 1.68−1.63 (m), 1.91−1.84 (m), 2.81 (s), 2.90 (s), 3.01−2.96 (m), 3.54−3.48 (m), 3.69−3.66 (m), 1.98 (br dd, J = 11 Hz, 5 Hz), 4.45−4.41 (m), 4.74 (dt, J = 9 Hz, 6 Hz), 5.23 (dd, J = 9 Hz, 3 Hz), 6.91 (br d, J = 7 Hz), 7.05 (t, J = 8 Hz), 7.36−7.18 (m), 7.48 (d, J = 8 Hz), 7.51 (t, J = 9 Hz), 8.90 (d, J = 9 Hz), 12.07 (s).

Reduction of the Triflate (3).[6] The crude triflate was dissolved in anhydrous DMF (2 mL), and to it was added 1 spatula tip of $Pd(OAc)_2$ and 1 spatula tip of DPPF (more than catalytic amounts). The mixture was heated to 60 °C, during which time it darkened in color. Et_3SiH (0.5 mL) was added and the mixture immediately turned black. The temperature was raised to 100 °C and held there for 10 min, after which time HPLC showed 30% conversion (method as above; t_R starting material = 5.52 min, t_R product = 5.00 min). Further aliquots of the reagents described above were added in the same order, and after heating 10 more min conversion was 65%. Repeating the sequence a third time gave 100% conversion, as determined by HPLC. The solution was cooled, and MeOH (5 mL) was added. Suspended materials were removed by filtration through a plug of Celite using MeOH as eluent. The filtrate was concentrated in vacuo to give a brown oil, which was chromatographed by reversed-phase HPLC, giving 9.0 mg (73% isolated yield over two steps) of pure **1** as a crystalline solid, which was identical by NMR to the natural material from fermentation.

Synthesis of Cycloaspeptide E from Methyl Alaninate Hydrochloride. 2-Nitrobenzoyl-L-Ala-OMe. To a cold (0−5 °C), well-stirred, nitrogen-blanketed suspension of methyl alaninate·HCl (1.4 g, 0.01 mol) in a mixture of triethylamine (3.0 mL, 0.022 mol) and DCM (25 mL) containing DMAP (∼50 mg) was added dropwise a solution of 2-nitrobenzoyl chloride (1.9 g, 0.01 mol) in DCM during 10 min, maintaining the reaction temperature at 0−5 °C. This mixture was stirred in the cold for 2.5 h, then diluted with DCM (40 mL) and water (25 mL). The organic phase was separated and washed successively with 1 N HCl (20 mL), saturated $NaHCO_3$ (25 mL), and brine (25 mL) and then dried (Na_2SO_4). Concentration left 2.3 g (99%) of the title compound as a colorless foam, which was pure enough to be used without further purification: ^{1}H NMR ($CDCl_3$) δ 1.69 (d, 3, J = 7 Hz), 3.92 (s,3), 4.94 (m, 1), 6.65 (d, 2, J = 6 Hz), 7.64−7.74 (m, 2), 7.82 (m, 1), 8.20 (d, 1, J = 8 Hz).

H-Ant-L-Ala-OMe. A solution of 2-nitrobenzyl-L-ala-OMe (1.0 g, 4.23 mmol) in ethanol (50 mL) was hydrogenated at 45 psi over 20% $Pd(OH)_2$/C catalyst for 4 h. The catalyst was removed by filtration through a bed of Celite and washed with ethanol (25 mL). The combined filtrate and wash was concentrated, leaving the title compound (1.0 g, quant.) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.50 (d, 3, J = 7 Hz), 3.78 (s, 3), 4.75 (m, 1), 5.51 (br s, 2), 6.64−6.69 (m, 3), 7.15−7.27 (m, 2), 7.39 (d, 2, J = 7 Hz).

Fmoc-L-MePhe-Ant-L-Ala-OMe. This compound was prepared using procedure A, but with a coupling reaction time of 2 h. From 1.7 g (4.25 mmol) of Fmoc-L-MePhe and 1.0 g (4.23 mmol) of Ant-L-ala-OMe, 1.5 g (60%) of the title compound was obtained as a colorless foam after chromatographic purification with 4% acetone in DCM as eluent: ^{1}H NMR ($CDCl_3$) δ 1.34 (d, 3, J = 7 Hz), 2.91 (s), 2.98 (s), 3.10 (m), 3.50 (m), 3.67 (s), 3.754 (s), 4.21−4.38 (m), 4.40−4.60 (m), 4.90 (m), 5.25 (m), 6.72 (m), 7.10−7.60 (m), 7.77 (m), 8.70 (m), 11.65 and 11.68 (s, total 1H).

H-L-MePhe-Ant-L-Ala-OMe. This compound was prepared using procedure B. From 0.6 g (1.01 mmol) of the protected tripeptide, 0.4 g (quant.) of the title compound as a colorless oil was obtained. This was used without further purification: ^{1}H NMR ($CDCl_3$) δ 1.52 (d, 3, J = 7 Hz), 2.17 (s), 2.34 (s), 2.76 (dd, J = 13, 9 Hz), 3.30 (m,2), 3.81 (s, 3), 4.80 (m), 6.74 (d, 2, J = 7 Hz), 7.12 (m, 1), 7.20−7.37 (m), 7.53 (m), 7.70 (m), 8.65 (d, 1, J = 8 Hz), 11.65 (s, 1).

Fmoc-L-Leu-L-MePhe-Ant-L-Ala-OMe. This compound was prepared using procedure A, but with a coupling time of 2.5 h. From 0.36 g (1.00 mmol) of Fmoc-L-Leu and 0.39 g (1.0 mmol) of l-MePhe-Ant-L-Ala-OMe, 0.4 g (56%) of the title compound as a colorless foam was obtained after chromatographic purification with 5% acetone in DCM as eluent: ^{1}H NMR ($CDCl_3$) δ 0.69 (d, J = 6 Hz), 0.93 (d, J = 6 Hz), 0.98 (d, J = 6 Hz), 1.47 (d, J = 7 Hz), 1.30−1.60 (m), 2.95 (s),

3.00 (m), 3.57 (dd, $J = 14$, 6 Hz), 3.75 (s), 4.18 (m), 4.35 (d, $J = 6$ Hz), 4.60 (m), 5.12 (d, $J = 9$ Hz), 5.47 (d, $J = 7$ Hz), 7.00−7.63 (m), 7.78 (d, $J = 8$ Hz), 8.64 (d, $J = 8$ Hz), 11.43 (s, 1).

H-L-Leu-L-MePhe-Ant-L-Ala-OMe. This compound was prepared using procedure B, with a reaction time of 1 h 30 min. From 0.53 g (0.74 mmol) of Fmoc-L-Leu-L-MePhe-Ant-L-Ala-OMe, 0.38 g (quant.) of the title compound as a colorless foam was isolated and used without further purification: ^{1}H NMR ($CDCl_3$) δ 0.69 (d, $J = 6$ Hz), 0.93 (d, $J = 6$ Hz), 0.98 (d, $J = 6$ Hz), 1.40−1.60 (m), 2.95 (s), 3.00−3.20 (m), 3.50−3.65 (m), 3.75 (s), 4.70 (m), 6.78 (d, $J = 7$ Hz), 7.10−7.30 (m), 7.60 (m), 8.60 (d, $J = 8$ Hz), 11.42 (s), 11.62 (s); LRMS (CI) *m*/*z* 497 (M + H, 45), 275 (100), 190 (40).

Fmoc-L-MePhe-L-Leu-L-MePhe-Ant-L-Ala-OMe. This compound was prepared using procedure A, but with a coupling time of 3.5 h. From 0.29 g (0.72 mmol) of Fmoc-L-MePhe and 0.36 g (0.72 mmol) of L-Leu-L-MePhe-Ant-L-Ala-OMe, 0.3 g (47%) of the title compound as a colorless foam was obtained after chromatographic purification over silica with 5% acetone in DCM as eluent: ^{1}H NMR ($CDCl_3$) δ 0.60 (d, $J = 6$ Hz), 0.82 (d, $J = 6$ Hz), 0.93 (d, $J = 6$ Hz), 1.45 (d, $J = 7$ Hz), 1.50 (m), 2.78 (s), 2.83 (s), 2.88 (s), 2.92 (m), 3.20 (m), 3.55 (dd, $J = 14$, 6 Hz), 3.75 (s), 4.05−4.40 (m), 4.70 (m), 4.90 (m), 5.53 (m), 6.10 (d, $J = 8$ Hz), 6.70 (m), 7.00−7.58 (m), 7.78 (d, $J = 8$ Hz), 8.60 (d, $J = 8$ Hz), 11.22 (s), 11.40 (s); LRMS (CI) *m*/*z* 880 (M + H, 20), 658 (100).

H-L-MePhe-L-Leu-L-MePhe-Ant-L-Ala-OMe. This compound was prepared using procedure B with a reaction time of 2 h. From 0.3 g (0.34 mmol) of Fmoc-L-MePhe-L-Leu-L-MePhe-Ant-L-Ala-OMe, 0.20 g (88%) of the title compound as a colorless foam was obtained after chromatographic purification with 4% MeOH in DCM as eluent: ^{1}H NMR ($CDCl_3$) δ 0.68 (d, $J = 6$ Hz), 0.92 (d, $J = 6$ Hz), 0.98 (d, $J = 6$ Hz), 1.48 (d, $J = 6$ Hz), 1.42−1.70 (m), 2.06 (s), 2.65 (m), 2.90−3.16 (m), 3.05 (s), 3.58 (dd, $J = 14$, 6 Hz), 3.80 (s), 4.74 (t, $J = 7$ Hz), 4.98 (m), 5.52 (dd, $J = 10$, 6 Hz), 6.80 (d, $J = 7$ Hz), 7.05−7.38 (m), 7.46−7.56 (m), 8.61 (d, $J = 7$ Hz), 11.35 (s); LRMS (CI) *m*/*z* 658 (M + H, 90), 436 (100).

H-L-MePhe-L-Leu-L-MePhe-Ant-L-Ala-OH. To a stirred solution of 0.195 g (0.29 mmol) of l-MePhe-L-Leu-L-MePhe-Ant-L-Ala-OMe in MeOH (4 mL), 0.34 mL of 1 N NaOH was added in one portion, and this mixture was stirred overnight at ambient temperature under nitrogen. HCl (1 N, 0.36 mL) was then added, and this mixture was stirred for 5 min. The solvent was evaporated to near dryness at reduced pressure and the residue diluted with DCM (50 mL) and water (5 mL). The organic phase was separated and washed with brine (6 mL) and then dried (Na_2SO_4). Concentration left 0.185 g (96%) of the title compound as a white powder, which was used as isolated: ^{1}H NMR (CD_3OD) δ 0.74 (d, $J = 6$), 0.92 (m), 1.34−1.64 (m), 2.42 (s), 2.78 (m), 2.90−3.10 (m), 3.00 (s), 3.05 (s), 3.20 (m), 3.45 (m), 3.80 (m), 4.58 (m), 4.90 (m), 7.00 (d, $J = 8$ Hz), 7.15−7.35 (m), 7.45 (m), 7.80 (d, $J = 8$ Hz), 8.22 (d, $J = 8$ Hz), 8.52 (d, $J = 8$ Hz).

Cycloaspeptide E (1) from Cyclization of L-MePhe-L-Leu-L-MePhe-Ant-L-Ala-OH (5) with 1-Propanephosphoric Acid Cyclic Anhydride. A solution of 133 mg (0.20 mmol) of l-MePhe-L-Leu-L-MePhe-Ant-L-Ala-OH in DCM (30 mL) was added dropwise over 6 h under nitrogen to a stirred solution of triethylamine (0.73 mL, 5.24 mmol), 1-propanephosphonic acid cyclic anhydride (T3P, 0.62 mL of a 50% solution in EtOAc, ∼1.0 mmol), and DMAP (∼2.5 mg) in DCM (125 mL). This solution was stirred for 48 h at ambient temperature, then concentrated to dryness at reduced pressure. The residue (340 mg) was chromatographed over 100 mL of flash silica using 3% MeOH in DCM as eluent to give 84 mg (67%) of **1**. LC-MS analysis of this showed 3−5% of a racemized product. Recrystallization from EtOAc−petroleum ether (1:4) gave pure product as colorless microcrystals, identical by ^{1}H NMR and LC-MS analysis to the natural material: mp 161−164 °C; $[\alpha]_D^{25}$ − 203 (*c* 0.1 MeOH).

Supporting Information Available: Representative NMR spectra (^{1}H and HSQC) of **1**; positive ionization electrospray mass spectrum of **1**; ultraviolet spectra of **1** and **2**; summary of cycloaspeptide-producing strains used in this study (Table S1); and comparison of insecticidal activity of **1** and **2** (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) De Jesus, A. E.; Steyn, P. S.; Van Heerden, F. R.; Vleggaar, R.; Wessels, P. L.; Hull, W. E. *J. Chem. Soc. Perkin Trans. 1* **1983**, *8*, 1847−1856.
(2) Kobayashi, R.; Samejima, Y.; Nakajima, S.; Kawai, K.; Udagawa, S. *Chem. Pharm. Bull.* **1987**, *35*, 1347−1352.
(3) Dalsgaard, P. W.; Larsen, T. O.; Christophersen, C. *J. Antibiot.* **2005**, *58*, 141−144.
(4) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(5) Dalsgaard, P. W.; Larsen, T. O.; Frydenvang, K.; Christophersen, C. *J. Nat. Prod.* **2004**, *67*, 878−881.
(6) Kotsuki, H.; Datta, P. K.; Hayakawa, H.; Suenaga, H. *Synthesis* **1995**, *11*, 1348−1350.
(7) Nakao, K.; Hamada, Y.; Shioiri, T. *Chem. Pharm Bull.* **1989**, *37*, 930−932.
(8) Lewer, P.; Graupner, P. R.; Gilbert, J. R.; Karr, L. L.; Chapin, E.; Duebelbeis, D. O.; Martin, L.; Fields, S.; Pearce, C. Poster 248, American Society of Pharmacognosy Meeting, Corvallis, OR, July 24−27, 2005.
(9) Frisvad, J. C.; Larsen, T. O.; Dalsgaard, P. W.; Seifert, K. A.; Louis-Seize, G.; Lyhne, E. K.; Jarvis, B. B.; Fettinger, J. C.; Overy, D. P. *Int. J. Syst. Evol. Microbiol.* **2006**, *56*, 1427−1437.
(10) Gargas, A.; DePriest, P. T. *Mycologia* **1996**, *88*, 745−748.
(11) Atlas, R. M. *Handbook of Microbiological Media*, 2nd ed.; CRS Press Inc.: Boca Raton, FL, 1997.

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