Kurstakins: A New Class of Lipopeptides Isolated from *Bacillus thuringiensis*

Yetrib Hathout,* Yen-Peng Ho, Victor Ryzhov, Plamen Demirev, and Catherine Fenselau

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

Received April 12, 2000

A novel class of lipopeptides was isolated from *Bacillus thuringiensis kurstaki* HD-1. Four compounds (1−4) were separated by high-performance liquid chromatography and their primary structures determined using a combination of chemical reactions and mass spectrometry. The four lipopeptides were found to have the same amino acid sequence, Thr-Gly-Ala-Ser-His-Gln-Gln, but different fatty acids. The fatty acyl chain is linked to the N-terminal amino acid residue via an amide bond. Each lipopeptide has a lactone linkage between the carboxyl terminal amino acid and the hydroxyl group in the side chain of the serine residue. Antifungal activity was demonstrated against *Stachybotrys charatum*.

Matrix-assisted laser desorbtion ionization (MALDI) can be successfully used to characterize and differentiate between different species or strains of *Bacillus* spores.[1] Analysis of a suspension of intact spores by MALDI provides a set of biomarkers with characteristic molecular masses for each *Bacillus* species studied. It has been concluded that these biomarkers are secondary metabolites synthesized by the microorganism. In general, these biomarkers have molecular masses ranging from 800 to 5000 Da. In a recent paper[2] using the same technique (MALDI-MS) to differentiate between vegetative cells, Lenders et al. were able to distinguish between several *Bacillus subtilis* strains, on the basis of characteristic molecular ions of biosurfactants, especially of the lipopeptide class.[2] Lipopeptides are synthesized by a number of *Bacillus* species and some *Pseudomonas* species, and they are classified according to their primary structure and their biological activity.[3−5] Surfactins,[6] for example, consist of a β-hydroxy fatty acid that is linked to the N-terminal amino acid of a heptapeptide by an amide bond. The carboxy end of the peptide is further linked to the β-hydroxyl group of the fatty acid via a lactone bond. Iturins[7] have structures similar to surfactins except that they contain a β-amino fatty acid called ituric acid instead a β-hydroxy fatty acid, and also different amino acid residues. A third group of cyclic lipopeptides includes polymixins and fengycins (also called plipastatins). This class consists of a 10 amino acid peptide, in which residues 3−10 form a cyclic octapeptide, via a lactone bond for fengycins[8] and a lactam bond for polymixins.[9] A branched fatty acid is connected to the N-terminal amino acid L-Glu in the case of fengycins and 2,4-diaminobutyric acid in the case of polymixins. The activity of these molecules is strongly correlated with their tertiary structure.[4] Other cyclic lipopeptides have been characterized with different ring sizes and fatty acyl chain lengths.[10] Bacterial lipopeptides have significant biomedical, agricultural, and environmental applications.[4,5]

In the present study a new class of lipopeptides was isolated from a culture of *Bacillus thuringiensis* subsp. *kustaki* HD-1 and their structure determined. Using a combination of mass spectrometry, chromatography, and chemical reactions, the primary structures of three lipopeptides, with molecular masses ranging from 878 to 906 Da, have been elucidated.

* Corresponding author. Tel.: 301-405-8619. Fax: 301-405-8415. E-mail: hathout@wam.umd.edu.

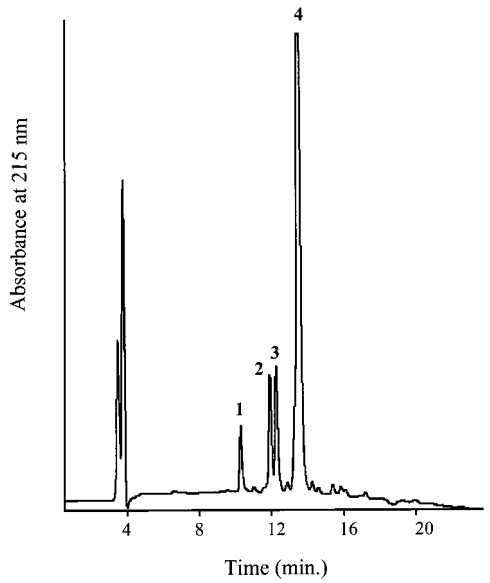


Figure 1. Reversed-phase chromatography of the lipopeptides obtained by washing *B. thuringinesis kurstaki* HD-1 spores.

Results and Discussions

Among the spores of *B. thuringiensis* strains tested in the present study, only *B. thuringiensis kurstaki* HD-1 provided a lipophillic biomarker with a molecular mass around 906 Da when analyzed by MALDI. This biomarker was invariably produced in three different growth media (CDSM, NB, and AK agar #2). However, the greatest amount was produced in AK agar #2, 15−20 μg/mg of spore, which is 20 times higher than with CDSM or NB.

In preliminary experiments, the total components washed from the surface of *B. thuringiensis kurstaki* spores were separated by HPLC. About 20 peaks were detected in this case. The peaks were collected and molecular masses were determined by MALDI. The lipopeptide fraction was found to be eluted with 60−70% of organic solvent B. Thus a bulk purification step was designed to eliminate more polar contaminants. As shown in Figure 1, a simple HPLC profile was obtained after C-18 cartridge prepurification. Peaks 1, 2, 3, and 4 were collected and their accurate molecular masses determined by FTMS (*m*/*z* 879, 893, 893, and 907, respectively). The masses of the MH^+ molecular ions differ by 14 Da, suggesting that they are homologous molecules.

10.1021/np000169q CCC: $19.00
Published on Web 09/15/2000

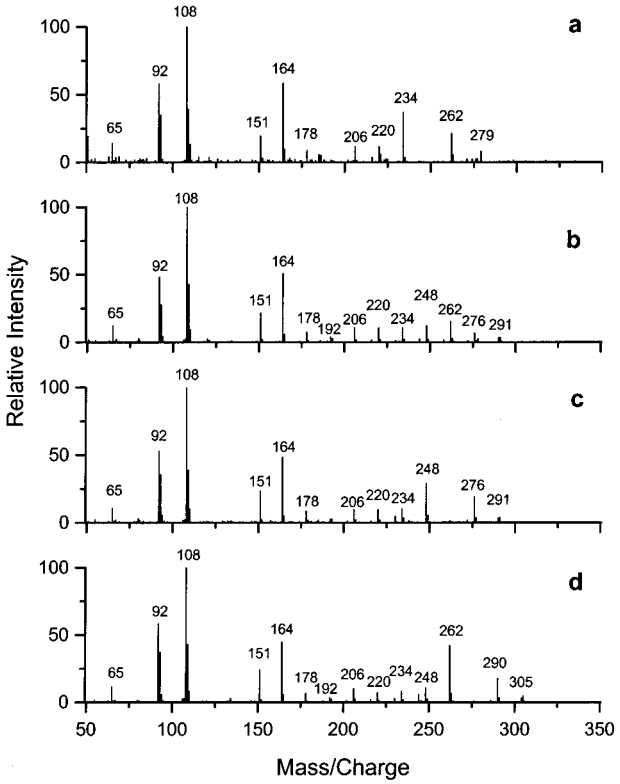


Figure 2. Electron impact mass spectra of the picolinyl derivatives of fatty acids cleaved from the purified lipopeptides: in (a) peak 1, (b) peak 2, (c) peak 3, and (d) peak 4.

The four compounds all produce a common fragment ion at *m*/*z* of 609, further supporting their homology. When collected separately, peaks 2 and 3 were found to have the same $[M + H]^+$ mass (893).

The four compounds isolated by HPLC were each subjected to acid hydrolysis, and the fatty acid moiety was derivatized as described in the Experimental Section. Electron impact spectra obtained using GC-MS are shown in Figure 2. All spectra exhibit odd mass molecular ions due to the nitrogen atom of the picolinyl moiety. The peaks at *m*/*z* of 92, 108(base peak), and 151(the McLafferty rearrangement ion) are characteristic of 3-picolinyl derivatives of fatty acids.[11,12] Starting from the molecular ions and progressing to lower masses, the fatty acid in Figure 2d shows a gap of 28 Da between the ions at *m*/*z* 290 and 262, while the other ions are spaced apart by 14 amu, accounting for methylenes (CH_2). The gap of 28 Da is characteristic for branched fatty acids with the methyl group branch located at the carbon atom just before the gap.[11,12] In this case the fatty acid derivative, characterized in Figure 2d, has an iso configuration. The corresponding free fatty acid is an 11-methyldodecanoic acid with a molecular mass of 214 Da.

The spectrum in Figure 2b, with no gaps of 28 Da between the fragment ions, is typical of straight chain fatty acids. This fatty acid is a straight chain dodecanoic acid (lauric acid). The spectrum of its isomer in Figure 2c, however, shows a gap of 28 Da between ions at *m*/*z* 276 and 248, and it is assigned as 10-methylundecanoic acid. These isomeric fatty acids cleaved from the two compounds with the same molecular mass of 892 Da may explain the resolution of peaks 2 and 3 in the chromatogram in Figure 1. The molecule bearing the branched fatty acid is retained in the reversed-phase column longer than the molecule bearing the straight chain fatty acid. Finally, the fatty acid in Figure 2a has 11 carbons with an iso configuration (9-methyldecanoic acid). These results clearly explain the difference of 14 Da observed for the purified biomarkers (Figure 1).

Amino acid analyses were carried out on ultrapure samples that had been passed three consecutive times through the HPLC column. Each sample was hydrolyzed with hydrochloric acid and its amino acid content analyzed as described in the Experimental Section. All three fractions contained the same amino acid residues (Thr, His, Ala, Gly, Ser, and Glx) with molar ratios of 1:1:1:1:1:2, respectively. We concluded that these biomarkers are heptapeptides linked to fatty acid chains.

Partial hydrolysis of the purified lipopeptide 4 (907 amu for the MH^+) using 1 M KOH for 1 h at room temperature gave a new product with an MH^+ of 925 Da (data not shown). The 18 Da gain in the mass is assigned to the hydrolysis of a lactone. After a longer hydrolysis (24 h) the molecular mass of the native lipopeptide shifted to 927 Da. This result suggested that the two Glx residues detected in the amino acid analysis were glutamine (Gln) residues in the native lipopeptide, which were converted to glutamate (Glu) residues during hydrolysis. The presence of a lactone linkage with the fatty acid and the presence of two glutamine residues give a theoretical monoisotopic mass (MH^+) of 906.495 Da. This is very close to the 906.510 Da measured by FTMS.

The homologous $[M + H]^+$ ions of masses 879, 893, and 907 all produced the same fragment ion at *m*/*z* 609 with the losses of 270, 284, and 298, respectively (Figure 1). Examination of the neutral fragments lost suggested that they contained fatty acyl moieties with differing number of CH_2 groups. Since the molecular masses of the fatty acids are known, the only amino acid residue among the ones found in the amino acid analysis that explains these neutral losses leading to the fragment ion at *m*/*z* of 609 Da is threonine. Thus, it was concluded that the fatty acyl moieties are linked to the threonine residue through an amide bond.

Figure 3 shows MS/MS spectra of the purified lipopeptide 4 obtained on the FTMS instrument before and after the lactone ring was opened. More information about the amino acid sequence is obtained from the spectrum of the open form (Figure 3b). The fragment ion at *m*/*z* 779 can be explained by the loss of 148 amu (Glu + 1 H) from the $[M + H]^+$ ion peak at *m*/*z* 927 (Figure 3b). The peak at *m*/*z* 650 corresponds to the loss of the two Glu residues ("b" ion series using the nomenclature for peptide fragmentation by mass spectrometry).[13] The fragment ion peak at *m*/*z* 414 corresponds to $[His\text{-}Glu\text{-}Glu]^+$, and that at *m*/*z* 501 corresponds to the fragment $[Ser\text{-}His\text{-}Glu\text{-}Glu]^+$. These fragments are a "y" ion series.[13] The fragment ion at *m*/*z* 629 corresponds to the loss of 298 from the $[M + H]^+$ ion, which is exactly the same neutral loss observed in the intact lipopeptide (Figure 3a). This result suggests that the lactone was located in the fragment ion of mass 609, which changes its mass to 629 after hydrolysis of the lactone and the conversion of the two Gln residues to two Glu residues. The only functional groups in this lipopeptide fragment that could be linked by a lactone are the carboxyl terminal of Gln and the hydroxyl group on the serine side chain. Thus, the lipopeptide is proposed to have a lactone ring composed of four amino acid residues (Ser-His-Gln-Gln), and the ring is further linked via an amide bond to the rest of the amino acids (Gly, Ala, and Thr). The sequence order of these residues will be discussed below.

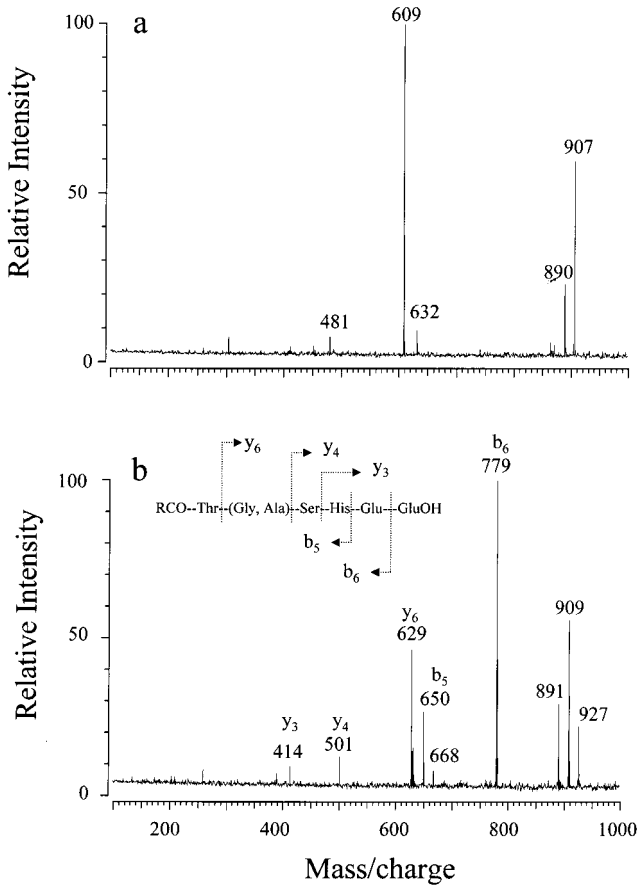


Figure 3. MS/MS spectra of purified lipopeptide 4 before (a) and after (b) hydrolysis of the lactone by 1 M KOH. The inset shows the origins of some of the fragment ions.

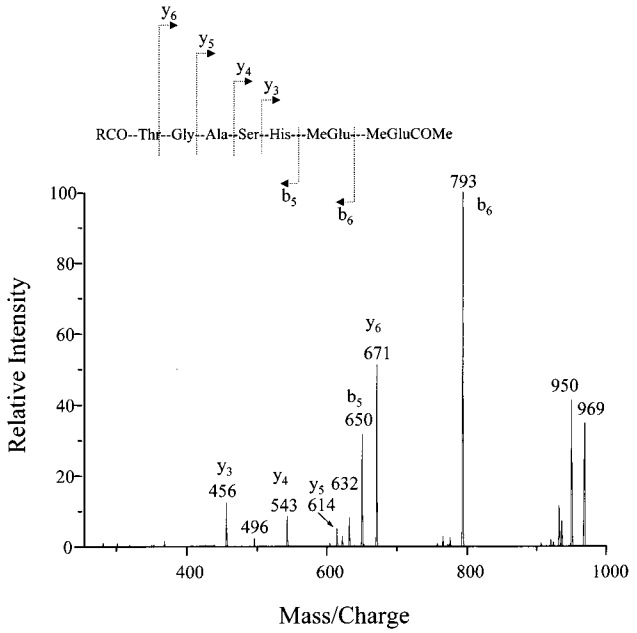


Figure 4. MS/MS spectrum of the open form of the lipopeptide 4 after methyl esterification of its free carboxyl groups.

The open form of the purified lipopeptide (MH^+ at *m/z* 927) was esterified as described in the Experimental Section. The resulting product was found to have an $[M + H]^+$ ion of 969 Da, 42 mass units higher than the starting material. This suggests that all three carboxyl groups (the C-terminal carboxyl group and the two carboxyl groups on the glutamate side chains) were converted to methyl esters. The tandem mass spectrum of this derivative is shown in Figure 4. Interpretation of the fragment ions is shown in the figure. Most of the fragment ions observed contain the His residue, because it is the most basic amino acid in the peptide. Loss of $(Me)_2$Glu from the $[M + H]^+$ ion of *m/z* 969 produces the fragment ion of *m/z* 793. Loss of the second MeGlu residue leads to an *m/z* 650 ion. The elimination of neutral fragments from the carboxyl terminus stops at the His residue. From the N-terminal end the loss of the 298 fatty acyl-Thr fragment is observed (*m/z* 671), as well as the loss of Gly (*m/z* 614), loss of Ala (*m/z* 543), and finally loss of Ser to form an ion of mass 456. These fragmentation patterns do not reflect the stereochemistry of the individual amino acids in the peptide.

We conclude that the most abundant lipopeptide isolated from *B. thuringiensis*, sbsp. *kurstaki* HD-1 spores has a molecular formula of $C_{41}H_{68}N_{11}O_{12}$ (**4**) (monoisotopic mass 905.495 Da) and that the primary structures are those shown in Figure 5.

Biocidal activity was tested against *Stachybotrys charatum* fungus. The purified lipopeptide mixture was found to inhibit fungal growth. A halo of inhibition 1 cm in diameter was observed around the center disk loaded with 80 μg of pure 905.5 Da lipopeptide, while the same amount of surfactin, a lipopeptide known to have no effect on fungal growth,[14] showed no inhibition at all. As a positive control, polymyxin B, previously assayed for antifungal activity as part of a mixture,[15] provided a similar 1 cm inhibition zone.

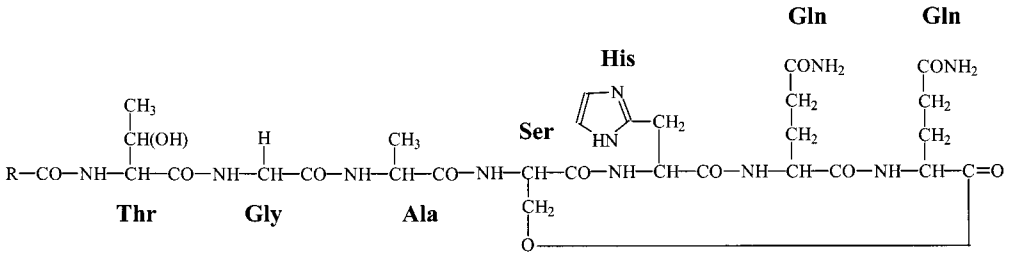
Thus, *B. thuringiensis kurstaki* HD-1 is a naturally occurring microorganism, which produces an insecticidal crystalline protein during sporulation.[16] Currently insecticides are manufactured under EPA regulations with *B. thuringiensis kurstaki* as the active ingredient.[17] In the present study we have shown that *B. thuringiensis kurstaki* HD-1 also produces a family of biologically active lipopeptides, which we named kurstakin. Kurstakin structure is closely related to the structure of other lipopeptides synthesized by *Bacillus* species, fungi, and yeasts. However, its amino acid composition incorporates a His residue, which is rare in the bacterial lipopeptides known today.[18−27]

The primary structure elucidation of this new class of active lipopeptides was made possible using a combination of chemical reactions and a variety of mass spectrometry techniques. This approach is valuable and convenient when dealing with a low amount of product for structural characterization.

Experimental Section

General Experimental Procedures. The lipopeptides were purified by reversed-phase chromatography using a Shimadzu instrument equipped with an LC-600 delivery pumps, an SPD-6 UV spectrophotometric detector, and a C-R6 A Chromatopac recorder. A Kratos MALDI 4 time-of-flight instrument with a curved field reflector, a 20 kV extraction voltage, and a 337 nm nitrogen laser was used for routine mass measurements. For accurate mass measurements a MALDI Fourier transform mass spectrometer equipped with a 337 nm nitrogen laser and a 4.7 T superconducting magnet was used. Tandem mass spectrometry analyses were performed either with the FTMS in the SORI-CAD mode or on a Finnigan LCQ ion trap mass spectrometer using Protona nanospray ion source. The analyte was electrosprayed by applying 1.2 kV to the silver-coated nanospray needle, and the capillary was heated at 180 °C. A Finnigan GC-MS instrument with an ion trap analyzer was used for fatty acid analysis.

Microorganisms. *Bacillus thuringiensis* subsp. *Kurstaki* HD-1 (ATCC 33679), *Bacillus thuringiensis* var. *galleria* (ATCC 29730), *Bacillus thuringiensis* Berliner (ATCC 13366,



1. R = $(CH_3)_2$-CH-$(CH_2)_7$- (HPLC peak 1)
2. R = CH_3-$(CH_2)_{10}$- (HPLC peak 2)
3. R = $(CH3)_2$-CH-$(CH_2)_8$- (HPLC peak3)
4. R = $(CH_3)_2$-CH-$(CH_2)_9$- (HPLC peak4)

Figure 5. Proposed structure of the lipopeptides isolated from *B. thuringiensis kurstaki* HD-1. R = $(CH_3)_2$−CH−$(CH_2)_7$− for the lipopeptide with molecular mass of 877.5 Da, HPLC peak 1. R = CH_3−$(CH_2)_{10}$− for the lipopeptide with molecular mass of 891.5 Da, HPLC peak 2. R = $(CH_3)_2$−CH−$(CH_2)_8$− for the lipopeptide with molecular mass of 891.5 Da, HPLC peak 3. R = $(CH_3)_2$−CH−$(CH_2)_9$− for the lipopeptide with molecular mass of 905.5 Da, HPLC peak 4.

ATCC13367, and ATCC 55172), and *Bacillus thuringiensis finitimus* (ATCC 19269) were purchased from the American Type Culture Collection (Rockville, MD). Typically, bacteria were grown in AK agar #2 plates (Becton & Dickinson, Cockeysville, MD). A high yield of spores (80−90%) was obtained after incubation at 37 °C for 1 day followed by 3 to 4 days incubation at room temperature. The spores were harvested from the agar plates using a rubber scraper, suspended in 50 mL of 1 M KCl/0.5 M NaCl solution, and recovered by centrifugation at 10000*g* for 10 min. The pellets were further washed 3 times with 50 mL of distilled water, lyophilized, and stored at −20 °C for further extractions. Production of the lipopeptides by *B. thuringiensis kurstaki* was also evaluated using a chemically defined spore medium (CDSM) and nutrient broth medium (NB).[1]

Screening for Lipopeptide-Producing Microorganisms. Colonies from each *B. thuringiensis* strain grown in agar plates were harvested using a sterile loop and suspended in 50 μL of acetonitrile/water/trifluoroacetic acid (TFA) (70:30:0.1, v/v/v). This suspension (0.3 μL) was deposited in the well of a MALDI sample slide and covered with 0.3 μL of 50 mM matrix solution of 4-methoxycinnamic acid in acetonitrile/water/TFA (70:30:0.1, v/v/v). The samples were analyzed in the positive ion mode using a Kompact MALDI 4 time-of-flight instrument (Kratos Analytical Instruments, Chestnut Ridge, NY) equipped with pulsed extraction and a nitrogen laser operating at 337 nm. Spectra were obtained in positive reflectron mode at an accelerating voltage of 20 kV.

Extraction and Purification of Lipopeptides. Depending on the amount of lipopeptide needed, 10−50 mg of dry spores was suspended in 5 mL of acetonitrile/water/trifluoroacetic acid (TFA) (90:10:0.1, v/v/v) and vigorously mixed using a vortex followed by mild centrifugation at 3000*g* for 10 min. The supernatant, containing components washed from the spore surface, was dried using a vacuum centrifuge. The dry extract was dissolved in 4 mL of acetonitrile/water/TFA (30:70:0.1, v/v/v) and applied to a cartridge column packed with 500 mg of C-18 silica. Retained material was washed with 10 mL of 0.1% TFA followed by 10 mL of acteonitrile/water/TFA (30:70:0.1, v/v/v). The lipopeptide fraction was eluted from the cartridge by 10 mL of acetonitrile/water/TFA (90:10:0.1, v/v/v) and dried. Further purification was performed by HPLC, using a semipreparative Jupiter C-8 column (Phenomenex, Torrance, CA) with 10 μm particle size and 250 × 10 mm (*l* × *w*) connected to a Shimadzu LC-600 delivery pump (Columbia, MD). The sample was dissolved in 500 μL of acetonitrile/water/TFA (30:70:0.1, v/v/v) and injected into the column. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.08% TFA in acetonitrile (solvent B). The compounds were eluted by a linear gradient of solvent B, developed from 30% to 70% in 20 min at a flow rate of 5 mL/min. Peaks with different retention times were collected and freeze-dried.

Quantitative Analysis. *B. thuringiensis kurstaki* HD-1 spores were grown in three different media (AK agar #2, CDSM, and NB). For extraction of the lipopeptides 100 mg of dry spores from each batch was used. The lipopeptides were purified using the semipreparative column as described above. Lipopeptide fractions were collected, dried under vacuum, and weighed using a Mettler AT250 microbalance (Mettler Instrument Co., NJ).

Mass Spectrometry. Molecular masses of compounds in HPLC fractions were routinely checked using the Kompact MALDI 4 time-of-flight instrument as described above.

Accurate mass measurements were obtained on the MALDI Fourier transform mass spectrometer (FTMS) with a 4.7 T superconducting magnet (IonSpec Co. Irvine, CA). The nitrogen laser fluence was estimated to be 40 mJ/cm^2. The mass accuracy was better than 20 ppm. Tandem mass spectrometry (MS/MS) experiments were performed either with the FTMS in the SORI-CAD mode or on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA). All experiments on the LCQ were performed using nanoelectrospray disposable needles (Protana A/S, Odense, Denmark). About 4 μL of the sample solution (methanol/water/acetic acid, 50:50:2, v/v/v used as solvent) was loaded into the needle. The voltage applied to this needle was 1.2 kV, and the estimated flow rate was 20−40 nL/min. The LCQ source temperature was 180 °C.

Amino Acid Analyses. About 5 μg of the purified lipopeptide was hydrolyzed in vacuo at 110 °C for 22 h in 100 μL of boiling HCl containing 1% phenol. Free amino acids were separated by ion-exchange chromatography using a Beckman Model 6300 amino acid analyzer (Fullerton, CA), operated with the manufacturer's program for analysis of protein hydrolysates with sodium buffers. The amino acids were quantified by determination of the respective peak areas at 570 nm for primary amines or 440 nm for proline, following a postcolumn reaction with ninhydrin. Calibration of the system was achieved through the use of a Beckman amino acid standard mixture. Norleucine was added as an internal standard.

Fatty Acid Analyses. About 100 μg of purified lipopeptide mixture was hydrolyzed with 200 μL of 6 M HCl at 150 °C for 18 h. After the solution was cooled, 200 μL of distilled water was added, and the fatty acids were extracted with ether (3 × 1 mL). The ether extracts were combined and evaporated to dryness under a stream of nitrogen. Fatty acid picolinyl ester derivatives were prepared according to the method described by Harvey.[28] Analysis of the picolinyl esters was performed on a Finnigan GCQ mass spectrometer (ThermoQuest, San Jose, CA). About 4 μL of sample was injected into the gas chromatograph equipped with an auto sampler and a 25 m × 0.25 mm DB5 capillary column (J & W Scientific, Folsom, CA). The temperature of the injector was set at 250 °C, and the

helium carrier gas flow was set at 40 mL min^{-1}. The column temperature was programmed from 180 to 280 °C at 10 °C/min.

Structural Analysis of the Lipopeptides. A series of chemical reactions were performed with the purified lipopeptides, and the reaction products were characterized by MS/MS or MS/MS/MS analysis. In each step, about 10−15 μg of the purified lipopeptide was used.

To determine the lactone linkage, the lipopeptide was dissolved in 200 μL of 1 M KOH and reacted overnight at room temperature. The excess KOH was removed by passing the solution through a C-18 cartridge column (100 mg). The trapped sample was washed with 2 × 4 mL of 0.1% TFA, then eluted from the cartridge by 4 mL of acetonitrile/water/TFA (90:30:0.1, v/v/v). After freeze-drying, the sample was dissolved in 100 μL of acetonitrile/water/TFA (70:30:0.1, v/v/v) and subjected to MS/MS analysis.

The open form of the purified lipopeptide was subjected to esterification of its carboxylic groups following a method previously described.[29] To dry open form of the pure lipopeptide, 200 μL of methanol containing 16% acetyl chloride was added and the reaction carried out for 1 h at room temperature. An aliquot of the reaction product was directly analyzed by mass spectrometry.

Antifungal Assay. About 200 μg of pure lipopeptide was dissolved in 200 μL of absolute EtOH, and 80 μL was deposited stepwise (20 μL each time) on the top of a blank 6 mm paper disk (Becton Dickinson, Cokeysville, MD). The same procedure was performed with surfactin (Sigma Chemical Co., Saint Louis, MO). As a control, a blank paper disk was loaded with 80 μL of ethanol. After ethanol evaporation under sterile conditions the disks were transferred to the top of PDA agar (Difco laboratories, Detroit, MI) plates, freshly inoculated with *Stachybotrys charatum* (kindly provided by B. Jarvis, University of Maryland, College Park, MD). Polymyxin B 300 U ready-to-use disks (Becton Dickinson, Cokeysville, MD) were also tested against this fungus. Incubation was carried out at 37 °C for 24 h. The fungus was handled with caution because of its ability to produce a toxin that is active on inhalation.[30]

Acknowledgment. We thank Kim Dudley and Danying Zhu for culturing the microorganisms used in this study, Dr. X. Christopher Yu (Chiron Corporation) for providing the amino acid analysis, and Dr. Bruce Jarvis for providing *S. charatum* fungus for the bioassay activity. This work was supported by contracts from the Defense Advanced Research Projects Agency (DARPA) and from the Johns Hopkins University Applied Physics Laboratory.

Supporting Information Available: FTMS spectra of peaks 1, 2, 3, and 4 from Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP000169Q