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Efficient Purification of the Biosurfactant Viscosin from *Pseudomonas libanensis* Strain M9-3 and Its Physicochemical and Biological Properties

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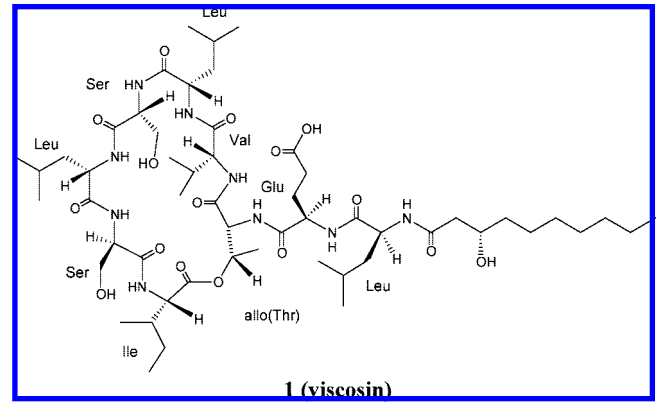
Viscosin (**1**), an effective surface-active cyclic lipopeptide, was efficiently recovered from *Pseudomonas libanensis* M9-3 with a simple purification protocol. A major pigment also obtained during this process was identified as phenazine-1-carboxylic acid. The critical micelle concentration (cmc) of viscosin was determined to be 54 mg L^{-1}, and the minimum surface tension between air and water at the cmc was 28 mN m^{-1}. Viscosin forms stable emulsions even at low concentrations (7.5 mg L^{-1}), and the conditional stability constant for a cadmium−viscosin complex was determined to be 5.87. The physicochemical properties measured for viscosin are similar to other well-studied biosurfactants such as rhamnolipid and surfactin. Viscosin inhibited migration of the metastatic prostate cancer cell line, PC-3M, without visible toxicity. These properties suggest the potential of viscosin in environmental and biomedical applications.

There is an increasing scientific and commercial interest in biologically produced surfactants. Thus far only two biosurfactants, namely, rhamnolipid and surfactin, have been examined in depth. This has resulted in our beginning to understand their roles in cell development, biofilm formation, and survival in adverse environments, suggesting that surface-active compounds may be an integral part of microbial survival strategies.[1−3] The same properties that make biosurfactants valuable for microbial survival also have potential for environmental, biotechnological, and pharmaceutical applications. Since only a limited number of natural biosurfactants are known, currently there is increasing effort to screen previously unexplored microbial sources for novel surface-active agents. These efforts have resulted in characterization of flavolipids from *Flavobacterium* sp. strain MTN 11,[4] trehalose dinocardiomycolates from *Rhodococcus opacus* ICP,[5] and surface-active molecules from probiotic *Lactobacillus*.[6] Herein we report the identification of bacterial isolate M9-3 obtained from a hydroponic greenhouse study[7] as *Pseudomonas libanensis*, a purification protocol for the biosurfactant viscosin (**1**) produced by *P. libanensis*, and its structure. A major pigment of this strain was also identified. Some physicochemical and biological characteristics of **1**, such as its critical micelle concentration (cmc), emulsification index, conditional stability constant for cadmium, and its ability to inhibit metastatic cancer cell migration, are also reported.

Viscosin was first isolated from a *Pseudomonas* sp. in 1951 as a result of its antibiotic activity against tubercle bacilli.[8] In general, cyclic lipopeptides (CLPs) like **1** consist of a cyclic oligopeptide polar moiety and a nonpolar fatty acid tail. Most CLPs have remarkably low surface activity and low cmc values,[9,10] and they are produced by a wide variety of bacteria in the genera *Bacillus*, *Pseudomonas*, and *Streptomyces*.[11−13] Different CLPs are distinguished on the basis of the size and amino acid content of the oligopeptide as well as the length and the number of fatty acids.[10,13] The structural diversity of CLPs and their widespread production in these three common soil bacterial genera are quite fascinating and suggest that they are important for survival and competitive interactions. Known functions of CLPs include antibiotic activity, iron sequestration, and antiviral and cytotoxic activities; some CLPs occur as cell wall components. The best studied CLP is surfactin, which is produced by *Bacillus* spp. Surfactin has been explored for a variety of activities including applications for remediation of metal-contaminated waste streams and soils.[14] Studies of surfactin and other CLPs have revealed that their biosynthesis is catalyzed by nonribosomal peptide synthetases (NRPS).[15] NRPSs are distinguished by their modular composition, where the modules generally correspond linearly to the amino acid sequence of an oligopeptide containing a total of seven amino acids: glu-leu-leu-val-asp-leu-leu.[16,17] In contrast, **1** contains a total of nine amino acids. Two (leu-glu) are linked to the fatty acid tail, and the remaining seven [val-leu-ser-leu-ser-ile-allo(thr)] form a cyclic oligopeptide.



1 (viscosin)

Results and Discussion

The bacterial isolate M9-3 (accession number EF076789) showed 99% homology to *Pseudomonas libanensis* (AF057645), which was previously isolated from spring waters in Lebanon.[18] *P. libanensis*

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is a fluorescent pseudomonad that has recently been described as part of the microbial community responsible for contributing to the volatile compound profile of processed cheese.[19] According to Dabboussi et al., *P. libanensis* can be differentiated from *P. aeruginosa*, which produces the well-known biosurfactant rhamnolipid, on the basis of pyocyanin production.[18] To confirm this and to eliminate the possibility that the biosurfactant produced by this isolate was rhamnolipid, the isolate M9-3 was screened for the *rhl*B gene[20] with negative results (data not shown). Therefore, the biosurfactant produced by M9-3 was purified to facilitate identification and physicochemical and biological evaluation. The growth conditions and solid-phase extraction (SPE) purification protocol used in this study resulted in a yield ranging from 125 to 150 mg L^{-1} of biosurfactant, reflecting recoveries of greater than 90%.

A white precipitate exhibiting surface activity was recovered using SPE and subsequently identified as the cyclic lipopeptide viscosin (**1**) by LREISMS (direct infusion), both in positive and in negative ion modes.[21] The presence of extracellular pigments in extracts of fluorescent pseudomonads has previously presented difficulties in the purification of their biosurfactants.[22] Extensive washing of the C-18 SPE column with 50−70% MeOH was effective in removing the pigments prior to elution of **1**. The major pigment associated with M9-3 production of **1** was greenish-yellow in color and was identified by ESIMS, FABMS, and FT-IR as phenazine-1-carboxylic acid.[23] A comparison of the UV absorption of the greenish-yellow pigment and the white precipitate containing **1** at 247 nm indicated the purity of **1** obtained by this purification procedure to be greater than 96%.

Although *P. libanensis* has not been previously reported to produce viscosin, CLP production of **1** has been reported from a variety of fluorescent pseudomonads including *P. syringae*, *P. tolaasii*, *P. fuscovaginae*, *P. corrugata*, *P. fluorescens*, and *P. putida*. The CLPs made by plant-associated *Pseudomonas* spp. have been broadly categorized into viscosin, amphisin, tolassin, syringomycin, and putisolvin[13] based on differences in the type and number of amino acids in the oligopeptide headgroup and the length and composition of the fatty acid tail. Many of these species have been noted for their antagonistic activity against different pathogenic fungi and for their role as plant pathogens.[13] For example, **1** has been reported to have a role in causing head rot of broccoli.[24] Nielsen et al.[25] reported that antifungal properties of *P. fluorescens* DR54 was due to the lipopeptide viscosinamide, which differs from **1** in the replacement of the second amino acid glutamate with glutamine. In general the CLPs made by *Pseudomonas* spp. have not yet been explored for their role in commercial applications.

It is likely that the SPE-based purification protocol for viscosin reported here could be extended to other CLPs as well. This type of rapid purification protocol is important for making these materials more readily available for commercial exploitation. Our procedure involves three steps: (i) separating the cells from the supernatant, (ii) acidification of the supernatant to precipitate **1**, and (iii) recovery of **1** using C-18 SPE. Application of this protocol led to >90% recovery of **1** with a purity of approximately 96%. This can be compared to the protocol of Laycock et al.,[24] which involved five steps with no yield reported. Our purification protocol can also be compared to a recent report of a four-step small-scale recovery of surfactin by ultrafiltration.[26] It was reported that ultrafiltration concentrated surfactin from an initial concentration of 1 g L^{-1} to 166 g L^{-1} with a recovery of 98%. The purity of the concentrated surfactin was 70%. In comparison with this method it appears that the SPE protocol reported here is simpler and produces material of higher purity.

There is relatively little information available on the physicochemical properties of viscosin and related CLPs produced by *Pseudomonas* spp. The minimum surface tension measured between air and water for the M9-3 viscosin was 27.5 mN m^{-1}. A cmc of

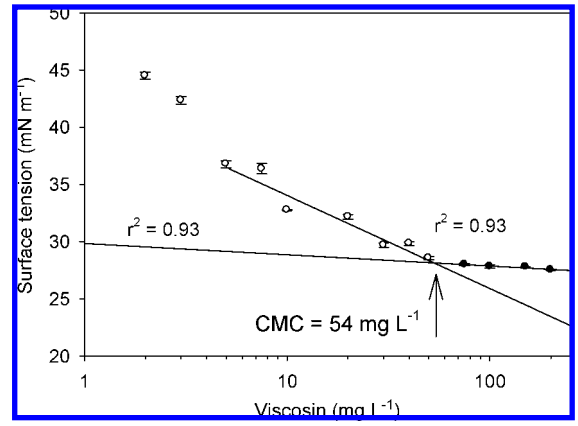

Figure 1. Effect of viscosin concentration on surface tension. The cmc was determined from the intersection of regression lines for (○) below cmc (5 to 50 mg L^{-1}) and (●) above cmc (75 to 200 mg L^{-1}). Each data point is the average and standard deviation of three replicate samples.

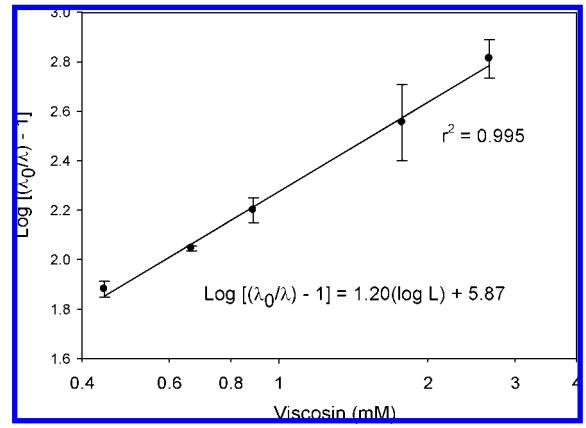

Figure 2. Determination of Cd^{2+} complexation constant and molar viscosin:Cd ratio using the ion exchange equilibrium method.

54 mg L^{-1} for viscosin was determined from a plot of the surface tension versus log viscosin concentration (Figure 1). Viscosin completely emulsified the oil layer (emulsification index = 1) even at the lowest concentration tested, 7.5 mg L^{-1}. The emulsion was stable after 1 week at room temperature. The viscosin−cadmium conditional stability constant (log K) was determined from the plot shown in Figure 2 to be 5.87. The number of biosurfactant ligands per complexed cadmium molecule (χ) was 1.20. These data indicate that **1** exhibited good surface activity and a low cmc that are comparable to other effective biosurfactants such as rhamnolipid and surfactin.[27] The reported cmc is different from values previously reported for **1**, 150 mg L^{-1} by Neu et al.[28] and 4 to 9 mg L^{-1} by Laycock et al.[24] The difference in reported cmc values may be due to differing biosurfactant purity or to the method used to estimate the cmc. The cmc in this report was estimated from Figure 1 after eliminating the two lowest concentration points from the linear regression because at low concentrations structural reorientation of the surfactant can occur at the air−water interface. Eliminating these two points caused the cmc estimate to increase from 44 to 54 mg L^{-1}. Viscosin exhibited complete and stable emulsification (EI = 1) at 7.5 mg L^{-1}. This can be compared to *P. fluorescens* strain 105, also reported to produce **1**, which had an EI equivalent to 3.[28] Flavolipid from *Flavobacterium* sp. MTN11 has been reported[4] to produce a stable emulsion at a concentration of 19 mg L^{-1}. Finally, the conditional stability constant for viscosin and cadmium was measured to be 5.87 (Figure 2). This is one log

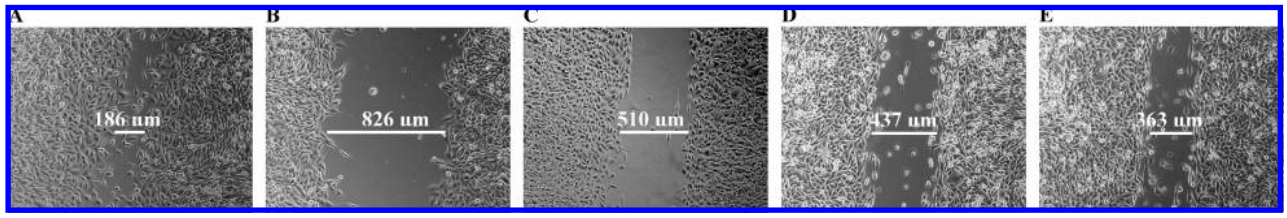

Figure 3. Effect of viscosin on migratory activity of the metastatic prostate cell cancer line PC-3M: (A) DMSO control (negative); (B) LY294002 control (positive) at 7.5 μM; (C) viscosin at 20.0 μM; (D) viscosin at 15.0 μM; (E) viscosin at 10.0 μM.

unit lower than the rhamnolipid−cadmium stability constant (6.89) reported by Ochoa-Loza et al. but several log units higher than organic acids such as acetic acid (1.2 to 3.2), oxalic acid (4.1), and citric acid (4.5).[29] The number of biosurfactant ligands per complexed cadmium molecules, χ, was measured to be 1.20. This non whole number value likely indicates the presence of a mixture of 1:1 and 2:1 viscosin:Cd complexes (ML^+ and ML_2) in solution, reflecting a stepwise formation of the ML_2 complex. Metal complexation studies with rhamnolipids have reported[29] $\chi = 1.91$ for rhamnolipid−Cd^{2+}. While this is the first report of a conditional stability constant for a CLP, surfactin has been used to evaluate metal removal from a soil contaminated with copper and zinc. A series of five washes with 0.25% (w/v) solution of surfactin in 1% NaOH was reported to remove 70% of the copper and 22% of the zinc.[14] Prabhukumar et al. have reported a conditional stability constant of 6.8 for cadmium−ELPH12.[30] The ELPs (elastin-like polypeptides) are chemically synthesized biopolymers capable of undergoing temperature-dependent phase transitions.

A variety of biological activities, including antibiotic[31] and inhibition of surface motility of root-pathogenic microfungi,[32] have been reported for **1**. The related cyclic lipopeptide surfactin has recently been found to inhibit proliferation of the colon carcinoma cell line LoVo by inducing pro-apoptotic activity and arresting cell cycle.[33] Therefore, it was of interest to evaluate **1** for its cell proliferation and cell migration inhibitory activities toward metastatic cancer cell lines. In a wound-healing assay (WHA)[34] for cell migration inhibition, **1** showed activity for the metastatic cancer cell lines PC-3M (prostate cancer) (Figure 3) and MDA-MB-231 (breast cancer) at 10 and 15 μM, respectively. In the MTT assay[35] for cell proliferation inhibition, **1** was found to be nontoxic to PC-3M up to 20 μM but was toxic (0% survival) to the MA-MB-231 cell line at 15 μM, the concentration at which it inhibited migration of this cell line. Microscopic examination of both types of cells after WHA supported the above findings. In order to further confirm that the PC-3M cell migration inhibition occurred at a subcytotoxic concentration, the PC-3M cells from the WHA with **1** were subjected to a flow cytometry-based cell viability assay;[36] cells were found to be completely viable (data not shown), suggesting that the PC-3M cell migratory inhibitory activity exhibited by **1** is not associated with its cytotoxicity. Further studies are currently in progress to understand the above difference in behavior of **1** toward the metastatic cancer cell lines PC-3M and MDA-MB-231.

Experimental Section

General Experimental Procedures. UV spectra were measured with a Hitachi U-2000 spectrophotometer. Direct-infusion LRESIMS were obtained in both positive and negative ion mode using a Finnigan Mat LCQ MS with an ionization voltage of 2.5 kV. HRFTMS were obtained using an IonSpec FTMS 4.7 T instrument in positive ion mode, and FABMS were obtained using a JEOL HX110A instrument in positive ion mode with direct sample infusion.

FTIR spectra were acquired using a Magna-IR model 550 spectrometer. Metal concentrations were measured using an Agilent 7500CE ICP-MS. Surface tension was measured using a surface tensiomat, model 21. Cell assays were performed using a Guava Easy Cyte flow cytometer.

Identification of *Pseudomonas libanensis M9-3*. The 16S rRNA gene from M9-3 was amplified using primers 27 F and 1492 R.[37] Each 25 μL reaction contained 0.5 μM of each primer, 0.2 mM of each dNTP, 1X buffer consisting of 10 mM Tris-HCl, 50 mM KCl, 2.0 mM $MgCl_2$ (pH 8.3), 5% DMSO, 1 U of *Taq* DNA polymerase, and 5.0 μL of cell lysate. The amplification program was 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.15 min, and a final extension at 72 °C for 10 min. The product was purified using a QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA) and stored at −20 °C. Successfully amplified 16S rRNA products were submitted to The University of Arizona Research Laboratories Genomic Analysis and Technology Core for sequencing using primers 518r, 518f, 1070r, and 1070f to obtain nearly full-length 16S rRNA gene sequences.[37] Similarity searches were performed by BLAST analysis (http://www.ncbi.nlm.nih.gov).

Biosurfactant Production and Purification. The M9-3 biosurfactant was produced and harvested from a mineral salt medium (MSM). A 1 L flask containing 250 mL of MSM was inoculated with a 1% (v/v) M9-3 culture that had been grown for 24 h at 200 rpm and 26 °C in Kay's medium. Kay's medium contained per L $(NH_4)H_2PO_4$ (3 g), K_2HPO_4 (2 g), glucose (2 g), $FeSO_4 \cdot 7H_2O$ (0.0024 g), and $MgSO_4 \cdot 7H_2O$ (1 g). MSM contained per L $NaNO_3$ (2.5 g), $MgSO_4 \cdot 7H_2O$ (0.4 g), NaCl (1.0 g), KCl (1.0 g), $CaCl_2 \cdot 2H_2O$ (0.05 g), and 85% H_3PO_4 (10 mL), adjusted to pH 7.2 with KOH pellets. One milliliter of a trace element solution was added containing per 100 mL $FeSO_4 \cdot 7H_2O$ (50 mg), $ZnSO_4 \cdot 7H_2O$ (150 mg), $MnSO_4 \cdot H_2O$ (150 mg), H_3BO_3 (30 mg), $CoCl_2 \cdot 6H_2O$ (15 mg), $CuSO_4 \cdot 5H_2O$ (15 mg), and $Na_2Mo_2O_4 \cdot 2H_2O$ (10 mg). The MSM was supplemented with 2% (w/v) glucose. The culture was harvested after 72 h growth at 200 rpm and 26 °C and centrifuged at 9000*g* for 15 min. The supernatant was acidified to pH 5.0 and allowed to stand for 24 h at 4 °C to allow precipitation of surface-active compounds. The bright yellow color of the supernatant indicated the presence of one or more pigments. Thus, the acidified supernatant (500 mL) was passed through a 25 mL solid-phase extraction (SPE) column containing 5 g of a high carbon loading (17%) C-18 end-capped support (Alltech, IL) using a vacuum manifold to maintain a constant drip. The pressure used was less than −15 kPa. After the sample was applied, the column was washed with nanopure water (pH 8) to remove trace salts and then extensively washed with an increasing gradient of MeOH/H_2O (pH 8) from 50% to 70% (v/v) MeOH until no yellow color was eluted from the column. The surface-active compounds were eluted from the column with 80% (v/v) MeOH, and the column was washed with 100% MeOH to recover any remaining bound material. The elution procedure was monitored by collecting 20 mL eluant samples and measuring the surface tension as an indicator of removal of surface-active compounds. Those fractions containing either yellow pigment or surface activity were air-dried for further analysis.

Structural Analysis of the Surfactant and Pigment. The pure concentrated surface-active compound (white flakes) was dissolved in 1:1 MeOH/H_2O and diluted before being analyzed by direct-infusion LREISMS in both positive and negative ion mode. MS results corresponded to those previously reported for viscosin[11] with main ions occurring at *m/z* 1126.5 ($M + H^+$), 1148.8 ($M + Na^+$), 1164.7 ($M + K^+$), and 1124.6 ($M - H^+$). HRFTMS confirmed the molecular formula, $C_{54}H_{95}N_9O_{16}$, for **1**, and the MS/MS analysis of the parent ion (at *m/z* 1126.5) produced fragment ions at *m/z* 1108.5, 843.3, 714.3, 631.4, 595.2, and 532.3. The yellow pigment was determined to be identical to phenazine-1-carboxylic acid[23] when analyzed by ESIMS, FABMS, and by FT-IR spectroscopy.

Determination of Surface Tension and Emulsification Index. Surface tension measurements were performed on triplicate serial dilutions of the biosurfactant (0 to 250 mg L^{-1}) to determine the cmc of the surfactant. The emulsification index (EI) was determined using

a modification of the method described by Willumsen and Karlson.[38] Six milliliters of surfactant (0, 7.5, 15, and 30 mg L^{-1}) was placed in screw-top glass test tubes (16 × 150 mm) in triplicate and overlaid with 10% (v/v) Pennzoil 10W-40. The height of the oil layer was measured, and then the tube was vortexed for 1 min to create an emulsion. The height of the emulsion layer was measured after 2 h, 24 h, and 1 week.

Cadmium Complexation. An ion-exchange technique was used to determine the conditional stability constant for **1** and cadmium.[29,39] All metal complexation experiments were performed in 0.01 M Pipes [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer solution (Sigma, St. Louis, MO) adjusted to pH 6.9 using 1 M NaOH. The resin used was sodium-saturated SP Sephadex C-25 (Pharmacia Biotech AB, Uppsala, Sweden), which was allowed to expand overnight in distilled H_2O, washed in distilled H_2O, washed several times in 0.01 M Pipes buffer, and then air-dried. For each experiment, 0.5 g of air-dried resin was transferred into triplicate 7 mL polypropylene scintillation vials containing 5 mL of Pipes buffer solution, viscosin (0 to 3000 mg L^{-1}), and cadmium (0.5 mM). Samples were shaken at 100 rpm on a rotary shaker for 2 h at room temperature to allow the mixture to reach equilibrium and then allowed to settle for at least 1 h. Cadmium concentrations in the supernatant were determined by ICP-MS using USEPA method 6020.[40] The cadmium−viscosin conditional stability constant, log *K*, and the number of ligands per cadmium−viscosin complex, χ (mol mol^{-1}), were calculated using the equilibrium complexation of cadmium with **1** and of cadmium with the cation-exchange resin as described previously.[29,39] This relationship can be applied only if the organic ligand (viscosin) is not bound by the ion exchanger and the metal solution concentration is small compared to that of the complexing agent. Preliminary investigation showed that **1** does not bind to the ion-exchange resin (data not shown).

Cytotoxicity Assay. The tetrazolium-based colorometric assay (MTT assay)[35] was used for the in vitro assay of cytotoxicity to NCI-H460 (non small cell lung cancer), MIA Pa Ca-2 (pancreatic carcinoma), MCF-7 (breast cancer), and SF-268 (CNS glioma). Doxorubicin and DMSO were used as positive and negative controls.

Wound-Healing Assay (WHA). For WHA the metastatic prostate cancer (PC-3M) cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 IU mL^{-1}), and streptomycin (50 μg mL^{-1}), and the metastatic breast cancer (MDA-MB-231) cells were cultured in DMEM/Ham's F-12 medium containing 10% fetal bovine serum and gentamycin (50 μg/mL). Both types of cells were grown in a 5% CO_2 atmosphere at 37 °C, and the cells were harvested at or above 80% confluence. The cells were plated onto sterile 24-well plates at a density of 70 000 cells per well for PC-3M and 150 000 cells per well for MDA-MB-231 and were allowed to recover for 48 and 24 h, respectively, until a confluent cell monolayer formed in each well (>90% confluence). Wounds were then inflicted to each cell monolayer using a sterile toothpick, media were removed, and the cell monolayers were washed once with PBS. Then fresh media were added to each well. Test samples (extracts, fractions, or beauvericin) were prepared in DMSO at different concentrations and added to the plates, each in duplicate along with the two controls, phosphotidylinositol (PtdIns) 3-kinase inhibitor LY294002[41] at 7.5 μM (positive control) and DMSO (negative control). The plates were incubated for 40 h (PC-3M) or 20 h (MDA-MB-231), during which the wells treated with DMSO had healed entirely and the wells treated with LY294002 and samples containing cell motility inhibitors had wounds present. All treatments, including the controls, were documented photographically. A treatment was considered active if there was a wound present in duplicate wells at the completion of the assay.

Flow Cytometry Assay.[36] Cells obtained directly from the wound-healing assays were collected with trypsin, washed twice with PBS, and stained with Guava PCA 96 ViaCount Flex reagent according to the manufacturer's instructions. Cells were assayed on a Guava Easy Cyte flow cytometer using EasyFit analysis to distinguish between viable and dead cells or debris.

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