Solenopsin A, a Venom Alkaloid from the Fire Ant *Solenopsis invicta*, Inhibits Quorum-Sensing Signaling in *Pseudomonas aeruginosa*

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In *Pseudomonas aeruginosa,* quorum-sensing (QS) signaling regulates the expression of virulence factors and thus represents an attractive new target for anti-infective therapy. In the present study, we investigated whether solenopsin A, a venom alkaloid from the fire ant, possessed agonistic or antagonistic QS signaling activity in *P. aeruginosa*. We evaluated the modulation of virulence factor expression and transcriptional levels of QS-regulated genes in *P. aeruginosa* by solenopsin A and demonstrated that solenopsin A efficiently disrupted QS signaling. Interestingly, exogenously added C_4–homoserine lactone (HSL), but not 3-oxo-C_{12}-HSL, restored *P. aeruginosa* QS signaling, suggesting that solenopsin A targets the C_4-HSL–dependent *rhl* QS system.

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is able to infect insects, plants, and humans [1]. Importantly, chronic *P. aeruginosa* infections are common in patients with cystic fibrosis, causing serious medical complications. The virulence of *P. aeruginosa* is regulated in a population density–dependent manner, a microbial process known as "quorum sensing" (QS). QS is a means of intercellular communication used by both gram-negative and gram-positive bacteria and is mediated by small, diffusible molecules termed "autoinducers" [2]. QS allows bacteria to control gene expression in a cell density–dependent manner, and, notably, genes regulated by QS encode a range of virulence factors, such as toxins and proteins involved in biofilm formation. Therefore, QS signaling systems might represent attractive new targets for antimicrobial therapy [3]. In fact, a number of studies aiming to control bacterial virulence via disruption of QS signaling using synthetic small molecules, natural products, or antibodies have been conducted [3–6].

P. aeruginosa possesses 2 LuxR/I-type QS systems, termed *"las"* and *"rhl,"* which use 2 distinct acyl-homoserine lactone (AHL) autoinducers, 3-oxo-C_{12}-HSL and C_4-HSL, respectively. The *las* QS signaling system has been shown not only to regulate the production of virulence factors but also to influence the *rhl* QS circuit [1]. However, it has been demonstrated that the *rhl* QS system itself plays a significant role in controlling the pathogenicity of *P. aeruginosa,* for example, through the production of pyocyanin and elastase B and the formation of biofilms. Most inhibitors targeting the *P. aeruginosa* QS signaling circuits reported to date have been structural mimics of its autoinducers [4]. Recently, a new immunotherapeutic approach has been reported that uses QS-quenching anti-AHL antibodies [6].

Solenopsin A (*trans*-2-methyl-6-undecylpiperidine; solenopsin), a venom alkaloid from the fire ant *Solenopsis invicta,* exhibits global structural similarity to 3-oxo-C_{12}-HSL, possessing a long hydrocarbon chain attached to piperidine, a nitrogen-containing heterocycle, via a chiral carbon (figure 1) [7]. In addition, it has been shown that both solenopsin and 3-oxo-C_{12}-HSL possess antimicrobial activity against gram-positive bacteria [8, 9]. Moreover, a recent study demonstrated that solenopsin and 3-oxo-C_{12}-HSL exert a wide range of biochemical effects on mammalian cells [7, 10]. Thus, in the present study, we investigated solenopsin for its potential to act as a QS signaling agonist or antagonist in *P. aeruginosa*. We demonstrate here that it efficiently suppresses QS signaling, resulting in a decrease in virulent factor production and biofilm formation. More interestingly, in contrast to our initial expectation, our data suggest that the main inhibitory effect of solenopsin might be exerted on *rhl* signaling.

Methods. PAO1-I, PAO1-O, and PDO 100 strains were provided by B. Iglewski (University of Rochester). Syntheses of solenopsin and its analogues have been described elsewhere [7]. Virulence factor assays, including biofilm analysis, were per-

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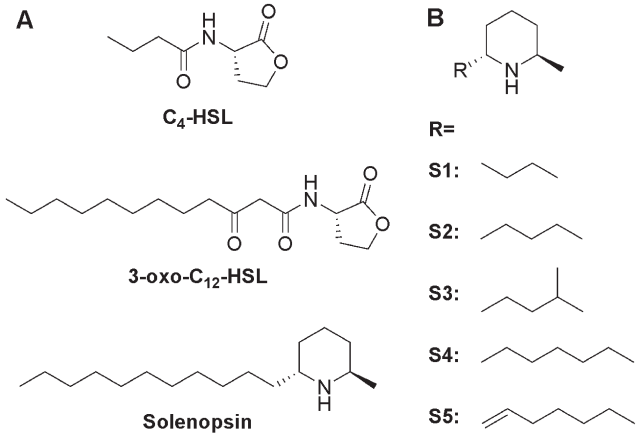

Figure 1. Structures of C_4–homoserine lactone (HSL), 3-oxo-C_{12}-HSL, solenopsin A, and solenopsin analogues (S1–S5).

formed following methods using PAO1-I, PAO1-O, or PDO 100 that have been described elsewhere [6]. For real-time polymerase chain reaction (PCR) analysis, fresh Luria-Bertani (LB) medium was inoculated with overnight-grown PAO1 (optical density $[OD]_{600}$, ~0.05), and cells were grown for ~3 h to an OD_{600} of ~ 0.5. Cells were diluted again into fresh LB containing solenopsin (50 μmol/L) and grown for ~5 h to an OD_{600} of ~0.6 and for ~6 h to an OD_{600} of ~2.2 for log- and stationary-growing phases, respectively. Cells were collected by centrifugation, total RNA was purified, and real-time PCR analysis was conducted as described elsewhere [5]. Nucleotide sequences of the primers used for real-time PCR analysis are as follows: *rpoD* forward, 5'-GGGCGAAGAAGGAAATGGTC-3'; *rpoD* reverse, 5'-CAGGTGGCGTAGGTGGAGAA-3'; *lasB* forward, 5'-TCATCACCGTCGACATGAACAGCA-3'; *lasB* reverse, 5'-AGTCCCGGTACAGTTTGAACACCA-3'; *rhlI* forward, 5'-TCTCTGAATCGCTGGAAGGGCTTT-3'; *rhlI* reverse, 5'-ATGGTCGAACTGGTCGAATTCCTG-3'; *lasI* forward, 5'-GCTCAAGTGTTCAAGGAGCGCAAA-3'; and *lasI* reverse, 5'-AGTGGTATCGAGAATTCGCCAGCA-3'.

Results and discussion. Pyocyanin, a QS-controlled virulence factor of *P. aeruginosa,* has been shown to cause serious tissue damage in chronic lung infection [11]. To examine whether solenopsin has an effect on AHL-mediated QS signaling in *P. aeruginosa,* pyocyanin production was investigated in *P. aeruginosa* in the presence or absence of solenopsin. To quantify the amount of pyocyanin produced, an extraction was con-

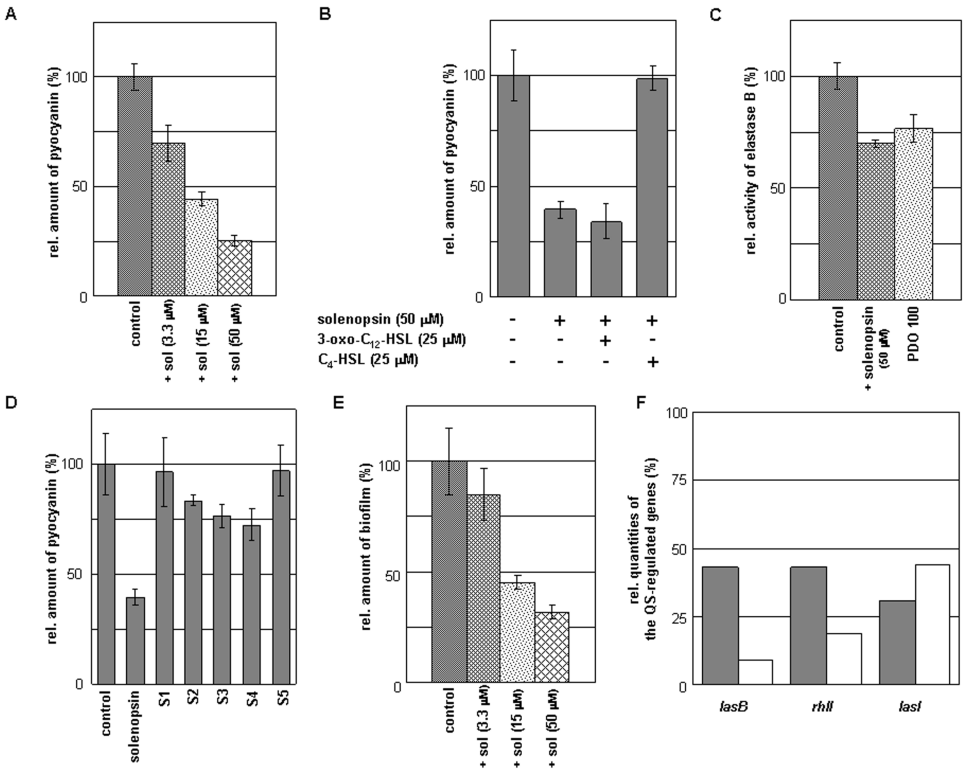

Figure 2. Virulence factor assays and real-time polymerase chain reaction analysis. *A,* Inhibition of pyocyanin production in PAO1-O by solenopsin. *B,* Effects of exogenously added acyl-homoserine lactone on the inhibition of pyocyanin production by solenopsin in PAO1-O. HSL, homoserine lactone. *C,* Elastase B production by PAO1-I in the presence of solenopsin (50 μmol/L) and the Δ*rhlI* strain PDO 100. *D,* Inhibition of pyocyanin production in PAO1-O by solenopsin and its analogues (50 μmol/L). *E,* Inhibition of biofilm formation of PAO1-I by solenopsin. *F,* Analysis of relative transcription levels of the indicated genes in the presence of solenopsin (50 μmol/L). For relative quantification, *rpoD* was used as a reference gene [12]. Gray bars represent the log-growing phase (optical density $[OD]_{600}$, ~0.6), and white bars represent the stationary-growing phase (OD_{600}, ~2.2). QS, quorum sensing.

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ducted directly from *P. aeruginosa* growth medium. The absorbance of the acidified aqueous solution containing pyocyanin was measured at 520 nm. As shown in figure 2*A*, a significantly lower amount of pyocyanin (≤50%) was found in the culture grown in the presence of solenopsin, indicating that solenopsin efficiently suppressed QS signaling of *P. aeruginosa* (median effective concentration, ~15 μmol/L). To investigate whether solenopsin acted as an AHL competitor, we tested whether the exogenous addition of the synthetic AHLs could restore pyocyanin production in *P. aeruginosa*. Surprisingly, our data (figure 2*B*) showed that exogenous C_4-HSL, but not 3-oxo-C_{12}-HSL, successfully competed with solenopsin and restored QS signaling in *P. aeruginosa,* suggesting that solenopsin might indeed target the *rhl* signaling system. This is especially noteworthy because there have been only a few reports on small-molecule inhibitors that target the *rhl* system. In light of these results, the modulation of elastase B production was investigated; elastase B is a metalloprotease whose expression is partly controlled by C_4-HSL QS signaling. Figure 2*C* demonstrates that solenopsin decreased elastase B production, although the effect (~30% decrease by 50 μmol/L solenopsin) is not as dramatic as that seen with pyocyanin. We reasoned that elastase B production might be less dependent on *rhl* QS signaling than pyocyanin synthesis. Hence, to support our rationale, we used a Δ*rhlI* mutant strain, PDO 100, in an elastase B assay and showed that 50 μmol/L solenopsin indeed suppressed elastase B production as efficiently as a genetically impaired mutant.

Because solenopsin inhibits the *rhl* QS system in *P. aeruginosa,* it is reasonable to hypothesize that molecules derived from solenopsin containing shorter acyl chains might have an increased antagonistic activity against C_4-HSL. Therefore, several solenopsin analogues (figure 1) prepared previously were investigated [7], but none of the analogues demonstrated increased QS inhibition (figure 2*D*).

As stated above, biofilm formation in *P. aeruginosa* is also regulated by QS signaling. Moreover, a recent study revealed that the *rhlI* mutant showed reduced biofilm formation compared with the wild-type strain, while a *lasI* mutant did not show significant impairment in biofilm formation [13]. Thus, solenopsin was also evaluated for its ability to modulate biofilm formation in *P. aeruginosa* via disruption of *rhl* QS signaling. *P. aeruginosa* was grown under static conditions in a polyvinyl chloride 96-well plate with peptone trypticase soy broth [14]. After 48 h of incubation without agitation, the plate was gently washed, and the biofilm was stained with crystal violet solution for relative quantification. Gratifyingly, our analysis demonstrated that the biofilm formation in *P. aeruginosa* was gradually reduced in the presence of solenopsin in a dose-dependent manner, but no changes were found with the solvent control, again indicating that solenopsin disrupts *rhl* QS signaling in *P. aeruginosa* (figure 2*E*).

Solenopsin has been shown to exert antimicrobial activity against gram-positive bacteria but possessed only weak toxicity toward gram-negative bacteria [8]. To verify that the observed decrease in virulence factor production and biofilm formation was not caused by growth inhibition, we monitored the growth of *P. aeruginosa* in the presence of solenopsin. Notably, no growth defect or retardation was observed over an 8-h period (data not shown), thus strongly suggesting that QS inhibition and the subsequent reduction of virulence factor production is indeed due to competition of solenopsin with C_4-HSL. Last, the transcription of 3 QS-controlled genes—namely, *lasB* (encoding elastase B) as well as *lasI* and *rhlI* (the 2 autoinducer synthase genes)—was analyzed by real-time PCR in the presence or absence of solenopsin [15]. Our data demonstrated that transcription of these QS-controlled genes was decreased ~2-fold (figure 2*F*), further confirming that solenopsin is a QS signaling inhibitor in *P. aeruginosa*.

In summary, we have demonstrated that solenopsin A, a venom alkaloid from the fire ant *S. invicta,* suppressed QS signaling in *P. aeruginosa,* resulting in modulation of virulence factor production as well as biofilm formation. An important implication of our data is that solenopsin might act mainly on the *rhl* QS signaling system as a competitor of C_4-HSL. We have recently introduced a QS-quenching immunotherapeutic strategy that might not be readily applicable to the *rhl* system, because the C_4-HSL molecule might be too diminutive to elicit a specific host immune response against it. In this regard, solenopsin could be a valuable starting point for the discovery of novel C_4-HSL antagonists and thus immensely valuable to the further development of therapeutic *rhl* signaling inhibitors.

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