Inhibitory effect of indole analogs against *Paenibacillus larvae*, the causal agent of American foulbrood disease

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Abstract

*Paenibacillus larvae*, a Gram-positive bacterium, causes American foulbrood (AFB) in honey bee larvae (*Apis mellifera* Linnaeus [Hymenoptera: Apidae]). *P. larvae* spores exit dormancy in the gut of bee larvae, the germinated cells proliferate, and ultimately bacteremia kills the host. Hence, spore germination is a required step for establishing AFB disease. We previously found that *P. larvae* spores germinate in response to l-tyrosine plus uric acid in vitro. Additionally, we determined that indole and phenol blocked spore germination. In this work, we evaluated the antagonistic effect of 35 indole and phenol analogs and identified strong inhibitors of *P. larvae* spore germination in vitro. We further tested the most promising candidate, 5-chloroindole, and found that it significantly reduced bacterial proliferation. Finally, feeding artificial worker jelly containing anti-germination compounds to AFB-exposed larvae significantly decreased AFB infection in laboratory-reared honey bee larvae. Together, these results suggest that inhibitors of *P. larvae* spore germination could provide another method to control AFB.

Key words: American foulbrood, spore germination, indoles
The *P. larvae* genome encodes putative Ger receptors similar to those found in *Bacillus* and *Clostridium* species. However, because direct interactions between Ger receptors and their cognate germinants have not been determined in any species, molecular probes have been used to map germinant-receptor interactions (Woese et al. 1958, Cortezzo et al. 2004, Abel-Santos and Dodatko 2007, Dodatko et al. 2009, Luu et al. 2011, Howerton et al. 2011). Using these approaches, we found that indole and phenol acted as antagonists of *P. larvae* spore germination (Alvarado et al. 2013).

The goal of the research reported here was to assess the efficiency of germination inhibition as a treatment strategy for AFB in vitro. A series of indole and phenol analogs were tested as inhibitors of uric acid/tyrosine-mediated germination of *P. larvae* spores. The half-maximal inhibitory concentration (IC50) of the best inhibitory indole analogs was calculated. We also tested the effect of indole analogs on *P. larvae* vegetative replication. Finally, indole inhibitors were tested for their ability to protect laboratory-reared honey bee larvae from AFB disease without toxic effects. These tests identified 3-chloroindole as a strong non-toxic inhibitor of spore germination, bacterial growth, and AFB disease development.

### Materials and Methods

#### Materials

Chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO) and WVR International (Radnor, PA). Dehydrated culture media components including yeast extract, Mueller–Hinton broth, and tryptic soy agar were purchased from BD Difco (Franklin Lakes, NJ) and Amresco (Solon, OH). *P. larvae* ERIC IV strain B-3685/ATCC 49843 was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). Lyophilized royal jelly was obtained from GloryBee Foods (Eugene, OR) and stored at -20°C until needed. Honey bee larvae food was prepared as described previously (Peng et al. 1992, Crailsheim et al. 2013).

**P. larvae** strain ATCC 49843 was grown on BD tryptic soy agar plates for 7 d under 5% CO2 at 37°C. The resulting bacterial lawns were collected by flooding with ice-cold deionized water and scraping plates using a cell spreader. The spores/vegetative cell mixture was pelleted by centrifugation 8,820 x g and resuspended in fresh deionized water. After three washing and centrifugation steps, spores were separated from vegetative cells and partially sporulated forms by centrifugation at 18,200 x g through a 20–50% Histodenz density gradient. The resulting spore pellet was washed five times with water as earlier and stored at 4°C for the duration of the experiment. Spore preparations were over 90% pure as determined by microscopic observation of Schaeffer–Fulton-stained samples (Schaeffer and Fulton 1933, Alvarado et al. 2013).

**Testing for Antagonists of *P. larvae* Spore Germination**

The decrease in optical density (OD) of a bacterial suspension is inversely proportional to spore germination (Powell 1950). To monitor spore germination in real time, changes in light diffraction intensity were monitored at 580 nm (OD at 580 nm) on a BioMate 5 (Thermo Electron Corporation, Waltham, MA) or a Tecan Infinite M200 (Tecan Group, Männedorf, Switzerland) spectrophotometer. Experiments were carried out in 96-well plates (200 µl/well).

In preparation for germination assays, *P. larvae* spore suspensions were washed three times with water, as earlier. Purified spores were then heat activated at 70°C for 30 min. Heat-activated spore aliquots were diluted with 0.1 M sodium phosphate buffer (pH 7) to an average OD at 580 nm [OD580] of 1.0. Spores were monitored for auto-germination for 30 min. Germination experiments were performed with spores that did not auto-germinate. Experiments were performed in triplicate with at least two different cultures. Relative OD values were derived by dividing each OD580 reading by its initial OD580 reading.

To test for antagonists of *P. larvae* spore germination, spore samples were first individually supplemented with 400 µM of individual indole or phenol analogs dissolved in dimethyl sulfoxide. Spore suspensions were incubated for 15 min at room temperature while monitoring changes in OD580. If no germination was detected, l-tyrosine and uric acid were added to final concentrations of 3,000 µM, and germination was monitored for 120 min. Germination percentage for each treatment was determined by dividing the final measured relative OD by the final measured relative OD of untreated controls.

The best inhibitors were further tested to determine their relative efficacy as anti-germinants. Spore samples were individually supplemented with individual indole analogs at concentrations of 0–1,600 µM. Germination rates were determined by linear fitting of changes in relative OD during the early time points. Germination rates for all conditions were divided by the uninhibited maximum germination rate obtained by treating spores with only germinants and are reported as percent germination. Percent germination was plotted against the log of inhibitor concentrations. The resulting sigmoidal curves were fitted using the four-parameter logistic function in SigmaPlot v.12 to calculate the half-maximal germination inhibitory concentrations (IC50).

### Testing for Indole Analogs Activity Against Proliferation of *P. larvae* Vegetative Cells

Overnight *P. larvae* cultures were diluted to an OD580 of -0.2, and 200 µl of the cultures were placed in 96-well plates. Indole analogs were added to *P. larvae* cells to a final concentration of 0, 50, 100, 150, 200, 500, 1,000, 1,500, or 2,000 µM. Cells were incubated at 37°C with shaking at 225 rpm for a 12-h period. OD was measured every 2 h on a BioMate 5 (Thermo Electron Corporation, Waltham, MA) or a Tecan Infinite M200 (Tecan Group, Männedorf, Switzerland) spectrophotometer. Experiments were performed in triplicate with two different cultures. Relative OD values were derived by dividing each OD580 reading by its initial OD580 reading.

#### Laboratory Rearing of Honey Bee Larvae

Experiments were conducted on larvae obtained from two colonies headed by naturally mated queens kept in the University of Nevada Las Vegas apiary according to standard industry practices (Honey Bee Genetics, Vacaville, CA). Artificial worker jelly (AWJ) was prepared an hour prior to use for larval rearing. d-glucose, d-fructose, and yeast extract were dissolved in warm autoclaved double-distilled water. This solution was mixed with lyophilized royal jelly for each treatment was determined by dividing the final measured relative OD by the final measured relative OD of untreated controls.

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First instar, 1-d-old honey bee larvae used in our experiments were collected from worker brood cells. Larval age was controlled by confining queens in a cage on empty frames. The queen was released after 24 h and the eggs were left in the hive until first instar larvae could be collected 3 d later. A group of 40 similarly aged
first instar, 1-d-old larvae were transferred to a six-well tissue culture plate containing 2.5 ml of the AWJ per well with a beekeeper’s grafting tool (Amazon, Seattle, WA). Plated larvae were grown at 35°C and 95% relative humidity following the published methods (Segur 1953, Crailsheim et al. 2013). The desired relative humidity and temperature were verified with an iButton data logger (Maxim Integrated, San Jose, CA). Two days after collection, honey bee larvae were transferred to individual wells on new 48-well plates containing 250 μl of fresh AWJ. From Day 3 until defecation at the pre-pupal stage, larvae were not relocated to reduce handling-related mortality.

Every 24 h, larvae were monitored for survival under a dissecting microscope. Larvae were classified as dead by the absence of movement or respiration, edema development, color change, and/or failure to pupate after 7 d (Evans 2004, Genersch et al. 2005). Dead honey bee larvae were immediately removed from their wells. In our hands, larvae fed AWJ reared from the first larval instar to the pre-pupal defecation had mortality below 3%.

**Analog Toxicity Assay**

Chronic exposure to indole analogs was performed to assess toxicity on honey bee larvae. Indole analogs were individually dissolved in autoclaved water to a 500 μM final concentration. Each indole analog stock solution was supplemented with d-glucose, d-fructose, yeast extract, and royal jelly (Supp Table 1 [online only]). Indole-supplemented AWJ was prepared an hour prior to use for larval testing. Larvae were placed on the indole-supplemented artificial diet as described earlier. Larval survival was determined every 24 h under a stereo microscope as described earlier. Each experiment contained three replicate trials with 30 larvae each, for a total of 90 larvae per treatment (Crailsheim et al. 2013).

**AFB Exposure Model**

A stock spore suspension with an OD540 of 1.0 was prepared in water (4.4 × 10⁸ spores per milliliter based on a microscopic count with a counting chamber). The stock spore suspension was diluted 1:100 with water, and 100 μl of the dilution (4.4 × 10⁶ spores) was added to 5 ml of AWJ. The spore-containing diet was prepared 1 h before use. Second instar (2-d-old larvae) were challenged with 2.2 × 10⁵ spores in 2.5 ml final volume (Supp Table 1 [online only]) of spore-containing diet. After spore exposure, third instar (3 d old) larvae were removed from wells containing the remaining spore-containing larval diets, cleaned using Kimwipes, and then moved to individual wells in 48-well plates containing fresh sterile 250 μl larval diets. Larval survival was determined daily as described earlier. Each experiment contained three replicate trials with 30 larvae, for a total of 90 larvae per treatment (Crailsheim et al. 2013).

**AFB Prevention by Indole Analogs**

The ability of indole analogs to prevent AFB infection was assayed by combining the toxicity and AFB exposure model protocols. Honey bee larvae were fed diets containing indole analogs at a 500 μM final concentration. The spore-containing diet was prepared 1 h before use and fed to second instar larva (2 d old) for a 24-h period. After spore exposure, third instar (3 d old) larvae were removed from wells containing the remaining spore-containing larval diets, cleaned using Kimwipes, and then moved to individual wells in 48-well plates containing fresh sterile 250 μl larval diets. Larval survival was determined daily as described earlier. Each experiment contained three replicate trials with 30 larvae, for a total of 90 larvae per treatment (Crailsheim et al. 2013).

**Survival Analysis**

Larval survival data were analyzed using Kaplan–Meier survival plots. The log-rank test from SigmaPlot v.12 was used to test for statistically significant differences between survival curves. The Holm–Šidák statistic was used to identify pairs of survival curves that are statistically significantly different from other pairs (P < 0.05).

**Results**

**Inhibition of P. larvae Spore Germination by Indole and Phenol Analogs**

We have previously shown that indole and phenol inhibited *P. larvae* spore germination (Alvarado et al. 2013). However, both indole and phenol (compounds 1 and 2) were weak inhibitors with sub-millimolar values of IC₅₀. To identify stronger inhibitors, the effect of 35 indole and phenol structural analogs (Fig. 1) on *P. larvae* spore germination in vitro was tested (Table 1) at a single concentration.

All phenol analogs (compounds 3–7) were weaker inhibitors of *P. larvae* spore germination than phenol. In fact, any tested modification to the benzene ring compromised inhibitory activity. In a similar manner, modification to the five-member ring of indole reduced the efficacy of the inhibitor on spore germination. Substitution of the N1 of indole for carbon (compound 8), sulfur (compound 9), or sulfonyl (compound 10) produced compounds that were unable to inhibit spore germination. Substitution of carbons for nitrogens at positions 2 (compound 11), 2,3 (compound 12), 3 (compound 13), or 7 (compound 14) of indole also abolished the inhibitory effect on spore germination. Benzothiazole (compound 15) only differs from indole by the addition of a sulfur group to position 3. However, compound 15 failed to prevent *P. larvae* spore germination. No increase in germination inhibition was observed with any benzothiazole derivatives tested (compounds 16–19). Allopurinol (compound 20), a purine analog that has functional groups found in both indole and uric acid, did not inhibit *P. larvae* spore germination as indole.

We had previously shown that methylation of indole did not increase anti-germination activity (Alvarado et al. 2013). Addition of acetyl, carbonyl, ethanamine, methoxy, carbosyl, hydroxyl, or amino functional group (compounds 21–27) reduced the inhibitory effect of indole on spore germination.

In contrast, indole analogs with electron-withdrawing groups (compounds 28–37) had increased inhibitory effects on *P. larvae* spore germination (Fig. 2 and Table 1). Quantitative analysis to compare the effectiveness of germination inhibition showed that the maximum inhibitory effect on *P. larvae* spore germination was obtained for indole analogs with halide and nitro group substituents (Table 2). Halide- and nitro-containing compounds were approximately four times as potent as indole. Additionally, cyano-containing compounds were approximately two to four times more potent than indole.

**Antimicrobial Activity of Indole Analogs on *P. larvae* Cells**

Tests of antimicrobial activity were performed with various concentrations (from 50 to 2000 μM) of 5-bromoindole, 5-chloroindole, 5-nitroindole, 6-bromoindole, and 6-chloroindole at. As shown with 5-bromoindole, indole analogs at final concentrations between 50 and 500 μM did not alter *P. larvae* cellular growth (Fig. 3). However, *P. larvae* growth was affected by higher concentrations of indole analogs. The most potent antimicrobial activity against *P. larvae* cells was observed in the presence of 6-bromoindole. The minimum inhibitory concentration at which no visible growth of *P. larvae* occurred
in medium was 2000 µM for all indole analogs tested. Antibacterial activity was greatly diminished at concentrations below 500 µM.

Indole Analogs do not Alter Honey Bee Larval Development

To test for toxicity, laboratory-reared larvae were fed AWJ supplemented with 500 µM of 5-bromoindole, 5-chloroindole, 5-nitroindole, 6-bromoindole, or 6-chloroindole (Fig. 4) from the first instar (1 d old) through the pre-pupation defecation period. Addition of 1500 µM of indole analogs resulted in a slight but statistically significant increase (Holm–Šidák method, $P < 0.05$) in larvae mortality in our hands. This was especially true for 6-chloroindole. However, even for 6-chloroindole, the survival rate was 92%, which is within the range of survival reported when larvae are simply manipulated during rearing (Peng et al. 1992, Huang 2009). Sub-lethal exposure to chemicals can in certain cases interfere with larvae behavior. However, under the conditions tested, larval mobility, size, respiration, and time to pupation were not affected (data not shown).

Indole Analogs Protect Honey Bee Larvae from AFB Disease

Second instar larvae-fed P. larvae spores started dying rapidly 4 d after challenge (Fig. 5). Only 22% of challenged untreated larvae survived to pupation. Honey bee larval survival was significantly higher (Holm–Šidák method, $P < 0.05$) for larvae that were fed with indole analogs after spore challenge. The highest survival percentages were observed in challenged larvae treated with 5-bromoindole (75%) or 5-chloroindole (65%).

Discussion

Previous work from our laboratory has shown the importance of using chemical probes to explore germination requirements that permit spores to exit their dormant stage (Dodatko et al. 2009; Alvarez et al. 2010; Howerton et al. 2011; Alvarado et al. 2013; Howerton et al. 2013a,b). We have identified germination inhibitors for B. anthracis, B. cereus, C. perfringens, C. sordellii, C. difficile,
and *P. larvae* (Akoachere et al. 2007; Alvarez and Abel-Santos 2007; Ramirez and Abel-Santos 2010; Dodatko et al. 2010; Howerton et al. 2011; Luu et al. 2011; Liggins et al. 2011; Howerton et al. 2013a,b). In this work, we expanded our search for indole analogs that would prevent *P. larvae* spore germination in vitro and reduced AFB disease in laboratory-reared larvae.

Most compounds tested in this study did not significantly reduce *P. larvae* spore germination in vitro. Changing the nitrogen or carbon atoms in the indole ring reduced the inhibitory effect of the molecule. Indeed, 13 out of the 35 compounds tested differed from indole only by two atoms but were unable to inhibit *P. larvae* spore germination. These results suggest that an intact indole ring is essential for binding at the putative germinant binding site.

The strongest inhibitors of *P. larvae* spore germination were indole analogs with electron-withdrawing groups (halide, cyano, and nitro). We hypothesize that indole analogs compete with the germinant uric acid for binding to the putative germination receptors. Both indole and uric acid structures are composed of two heteroaromatic rings. Electron-withdrawing groups in the most active indole analogs may have improved interaction with the putative germination receptor by mimicking the carbonyl groups found in uric acid.

Uric acid is present at saturating concentrations inside the gut of honey bee larvae, resulting in the formation of large crystals (Winston 1987, Yadav 2003). Similarly, the concentration of tyrosine in the larval gut has been estimated to be in the millimolar range (Liming et al. 2009). Indeed, the concentration of uric acid plus l-tyrosine used to

### Table 1. Percent germination of *P. larvae* spores (±SE) after treatment with indole and phenol analogs

| Compound | Germination (%) | SE  | Compound | Germination (%) | SE  |
|----------|----------------|-----|----------|----------------|-----|
| Control  | 100.0          | 1.9 | 01       | 56.8           | 2.9 |
| 02       | 47.6           | 3.6 | 03       | 97.6           | 2.7 |
| 04       | 99.2           | 1.6 | 05       | 100.4          | 1.4 |
| 06       | 98.9           | 1.9 | 07       | 100.1          | 1.5 |
| 08       | 97.7           | 2.3 | 09       | 91.3           | 8.9 |
| 10       | 96.3           | 2.0 | 11       | 91.7           | 3.9 |
| 12       | 94.0           | 4.7 | 13       | 93.9           | 5.5 |
| 14       | 101.3          | 2.6 | 15       | 92.8           | 3.1 |
| 16       | 92.9           | 9.3 | 17       | 93.7           | 3.6 |
| 18       | 89.1           | 1.9 | 19       | 98.4           | 4.1 |
| 20       | 80.6           | 1.9 | 21       | 77.3           | 6.4 |
| 22       | 87.1           | 6.9 | 23       | 93.6           | 5.0 |
| 24       | 70.3           | 2.5 | 25       | 99.0           | 1.0 |
| 26       | 81.4           | 8.8 | 27       | 101.7          | 5.7 |
| 28       | 24.8           | 9.8 | 29       | 37.1           | 14.4|
| 30       | 1.5            | 0.5 | 31       | 0.0            | 0.5 |
| 32       | 50.2           | 10.3| 33       | 0.3            | 0.2 |
| 34       | 1.2            | 0.5 |
| 35       | 0.6            | 0.1 |
| 36       | 0.0            | 0.3 |
| 37       | 17.4           | 1.4 |

*P. larvae* spores were incubated with 400 μM of an indole or phenol analog (Fig. 1) for 15 min prior to the addition of 3,000 μM l-tyrosine and 3,000 μM uric acid. Germination rates for all conditions were divided by the uninhibited maximum germination rate obtained by treating spores with 3,000 μM l-tyrosine plus 3,000 μM uric acid (control) and are reported as percent germination. Standard errors (SE) were calculated from at least six independent measurements.

![Fig. 2.](image-url) *P. larvae* spore germination in the presence of 5-bromoindole and 5-chloroindole. (A) *P. larvae* spores were incubated with germinants and supplemented with 0 μM (open circle), 40 μM (filled circle), 80 μM (open square), and 200 μM (filled square) of 5-bromoindole. (B) *P. larvae* spores were incubated with germinants and supplemented with 0 μM (open circle), 20 μM (filled circle), 100 μM (open square), and 200 μM (filled square) of 5-chloroindole. Data are shown every 5 min for clarity. Spore germination was measured by a decrease in the relative OD over time. Error bars represent standard error obtained from at least six independent measurements.
identify *P. larvae* germination inhibitors in this study was 3000 µM. We identified five indole analogs (5-bromoindole, 5-chloroindole, 5-nitroindole, 6-bromoindole, and 6-chloroindole) that had IC₅₀ values ~5–20 times lower than indole is spore germination inhibition assays while having very low antibiotic activity. These results are encouraging because utilizing antibiotic compounds for AFB treatment would certainly alter the microbiome and honey bee health. The five indole analogs selected not only prevented *P. larvae* spore germination in vitro but protected honey bee larvae from AFB disease at concentrations below the toxicity threshold. These five indole analogs are similar in their protective properties due to their common structural features. Thus, as the previous studies suggest, indole and indole analogs can serve as protective compounds against infectious organisms (Ueno et al. 2005, Kim et al. 2011, Alvarado et al. 2013). Because of our intervention, these larvae entered their pupation stage despite AFB infection.

**Conclusion**

AFB, a bacterial disease of honey bee larvae, is particularly troublesome because the infectious agent is the bacterial spore of *P. larvae* (Genersch 2010). Bacterial spores are resistant to extreme temperatures, antibiotics, and disinfectants. Spores can remain dormant for years until they can revert to vegetative cells (Alippi 1995, Dobbelare et al. 2001, Lodesani and Costa 2005). In this work, we tested the hypothesis that inhibiting *P. larvae* spore germination using indole analogs will result in decreased AFB disease in laboratory-reared honey bee larvae. We found that halide-substituted indoles prevented *P. larvae* spore germination in vitro and AFB disease in laboratory-reared larvae, suggesting that compounds like 5-chloroindole can be tested to develop a cheap, easily administered, and non-toxic treatment.

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**Table 2. Concentration of indole analogs that reduces *P. larvae* spore germination by half (IC₅₀ ± SE)**

| Compound | Name              | IC₅₀ (µM) |
|----------|-------------------|-----------|
| 2        | Indole            | 370 ± 20  |
| 29       | 4-Cyanoindole     | 200 ± 20  |
| 30       | 5-Bromindole      | 60 ± 70   |
| 31       | 5-Chloroindole    | 30 ± 10   |
| 32       | 5-Fluorindole     | 120 ± 20  |
| 26       | 5-Hydroxyindole   | 550 ± 170 |
| 33       | 5-Nitroindole     | 70 ± 10   |
| 34       | 6-Bromoindole     | 60 ± 60   |
| 35       | 6-Chloroindole    | 20 ± 0    |
| 36       | 6-Cyanoindole     | 110 ± 10  |
| 37       | 6-Fluorindole     | 120 ± 10  |

*P. larvae* spores were incubated with various concentrations of indole analogs for 15 min prior to addition of 3000 µM L-tyrosine and 3000 µM uric acid. IC₅₀ was calculated by plotting percent germination versus indole analog concentration for each condition. Standard errors (SE) were obtained from at least six independent measurements.

**Fig. 3.** Bacteriostatic activity of 5-bromoindole on *P. larvae* cells. *P. larvae* vegetative cells were grown in medium supplemented with 0 µM (open circle), 500 µM (filled circle), 1000 µM (open square), and 2000 µM (filled square) of 5-bromoindole. Bacterial growth was monitored over a 14-h period. Relative OD₅₀ values were obtained by dividing each data point by the initial OD.

**Fig. 4.** Toxicity of indole analogs on honey bee larvae. Honey bee larvae were reared in larval diet supplemented with water (open circle), 500 µM of 5-bromoindole (filled circle), 500 µM of 5-chloroindole (open square), or 500 µM of 6-bromoindole (open triangle), or 500 µM of 6-chloroindole (filled triangle). Surviving larvae were counted daily. Differences between survival curves were determined using Kaplan–Meier analysis, log-rank test (χ²=19.422, df = 5, P < 0.002), and the Holm–Šidák method (P < 0.05).

**Fig. 5.** Effects of indole analogs on AFB disease. Honey bee larvae were challenged with *P. larvae* spores in larval diet supplemented with water (crosses), 500 µM of 5-bromoindole (filled circles), 500 µM of 5-chloroindole (open squares), 500 µM of 5-nitroindole (filled squares), 500 µM of 6-bromoindole (open triangles), or 500 µM of 6-chloroindole (filled triangles). Unchallenged larvae (open circles) were used as experimental control and showed >95% survival. Differences between survival curves were determined using Kaplan–Meier analysis, log-rank test (χ²=194.484, df = 6, P < 0.001), and the Holm–Šidák method (P < 0.05).
Five indole analogs were identified that were potent inhibitors of spore germination in vitro, non-toxic to vegetative cells, non-toxic to larvae, and able to protect honey bee larvae from AFB disease. In every case, active indole analogs contained an electron-withdrawing group that made the indole ring electron-poor. We hypothesize that indole analogs interact with a putative electron-rich binding site that normally recognizes the carbonyl-rich uric acid germinant.

The larvae gut contains bacteria known to inhibit P. larvae vegetative growth (Evans 2004, Evans and Armstrong 2006, Yoshiyama and Kimura 2009, Forsgren et al. 2010). These bacteria should be preserved to avoid complications due to antimicrobial treatment with tetracycline (Raymann et al. 2017). Tetracycline treatment reduces the size and diversity of the microbiome observed in healthy honey bees. Furthermore, there is a correlation between the tetracycline use and a reduction in survival of bees exposed to opportunistic pathogens. The results of our work suggest that substituted indole analogs have weak antibiotic activity and should not affect the gut microbiome of the larvae.

It is improbable that P. larvae will develop resistance to indole analog treatments. One reason is that the dormant stage of the microorganism is targeted, rather than vegetative cells. Furthermore, if P. larvae alters the germination receptors, they might never exit dormancy or would germinate in an environment not suited for this fastidious organism. Thus, we will continue to identify compounds that block P. larvae germination receptors.

Developing a suitable inhibitor delivery system for honey bee colonies is the final step toward a potential disease treatment. Ideally, the treatment substances would be ingested, be effective at low concentrations, and degrade prior to contaminating honey stores. Honey bees are often fed sugar–water mixtures, sugar powder, or patties infused with treatments (Elzen et al. 2002, Yoder et al. 2014). In the colony, both nectar and pollen are eaten by bees performing larval care (nurse) duties (Craisheim et al. 1992, Grüter and Farina 2007). Once ingested by the nurse bees, the compounds can be delivered to individual honeycomb cells. Hence, nurse bees, normally a vector for the spread of P. larvae spores, could be used to prevent AFB (Gillard et al. 2008, Lindström et al. 2008, Garbian et al. 2012). The compounds identified need to work at low concentrations so that they cannot accumulate in the hive and the AFB treatment remains cost-effective. Similarly, the half-life of the compounds needs to be limited if the microorganism is targeted, rather than vegetative cells. Furthermore, if P. larvae alters the germination receptors, they might never exit dormancy or would germinate in an environment not suited for this fastidious organism. Thus, we will continue to identify compounds that block P. larvae germination receptors.

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