Larvae of the parasitoid wasp *Ampulex compressa* sanitize their host, the American cockroach, with a blend of antimicrobials

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Food resources contaminated with spoilage or pathogenic microorganisms pose severe problems to all higher organisms. Here, we describe a food-hygienic strategy of the emerald cockroach wasp *Ampulex compressa*. The wasp larvae develop on and inside the American cockroach *Periplaneta americana*, a host that can harbor various putrefactive microbes, as well as human and insect pathogens. From *P. americana*, we isolated the Gram-negative bacterium *Serratia marcescens*, which is a potent entomopathogen that can rapidly kill insect larvae. It is also known as a food contaminant and as an opportunistic human pathogen. Using behavioral observations and chemical analyses, we demonstrated that *A. compressa* larvae impregnate their cockroach hosts from inside with large amounts of an oral secretion containing a blend of γ-lactones and isocoumarins with (*R*)-(-)-mellein [(*R*)-(-)-3,4-diydro-8-hydroxy-3-methylisocoumarin] and micromolide [(4*R*,9*Z*)-octadec-9-en-4-olide] as dominant components. We fractionated hexane extracts of the secretion and investigated the antimicrobial properties of the fraction containing the lactones and isocoumarins, as well as of synthetic (*R*)-(-)-mellein and micromolide, against *S. marcescens* and a Gram-positive bacterium, *Staphylococcus hyicus*, in broth microdilution assays. The test fraction inhibited growth of both tested bacteria. The activity of the fraction against *S. marcescens* was explained by (*R*)-(-)-mellein alone, and the activity against *S. hyicus* was explained by the combined action of (*R*)-(-)-mellein and micromolide. Our data suggest that the specific combination of antimicrobials in the larval secretion provides an effective front-line defense against the unpredictable spectrum of microbes that *A. compressa* larvae may encounter during their development inside their cockroach hosts.

antibiotic | food hygiene | jewel wasp

Microbe-contaminated food poses severe threats to all animals from invertebrates to vertebrates, including humans. Microbial decomposers not only may cause the food to rot but also are often responsible for serious, sometimes even lethal, diseases when ingested with the food (1–4). Food hygiene is thus of vital importance to avoid potentially severe hazards associated with microbe-laden food resources.

Insects are increasingly recognized as sources of microbial food contaminations (5). In particular, cockroaches, such as the peridomestic American cockroach *Periplaneta americana* (Blattaria, Blattidae), are suspected vectors of food spoilage and pathogenic microbes (6, 7). Owing to their unsanitary lifestyle, they can pick up, carry, and transfer a plethora of bacteria and fungi (6–14) and hence represent a potential health problem to humans, as well as wild and domestic animals.

Animals that feed on cockroaches are especially at risk for acquiring infections from their contaminated food. One of these species is the emerald cockroach wasp *Ampulex compressa* F. (Hymenoptera, Ampuliciae), which relies on cockroaches such as *P. americana* for food for its offspring (15). The *A. compressa* female attacks a cockroach, stings it, and drags the then-docile host to a hiding place, where it glues an egg to the coxa of one of the mesothoracic legs of the cockroach. When the wasp's egg hatches, the larva first imbibes hemolymph through a hole in the thoracic cuticle of the still living cockroach. About 7 d after oviposition (at 27 °C), the larva moves inside the cockroach and feeds on the interior organs, causing the death of the host. During this time, the host cockroach represents both the food and the microenvironment of the larva. After eroding the cockroach almost completely, the larva spins itself into a cocoon inside the cockroach carcass, pupates, and ecloses as an adult about 6 wk after oviposition.

During the relatively long period of development and metamorphosis inside the cockroach, the larvae and pupae of *A. compressa* have to cope with competitive, putrefactive microorganisms that can degrade their larval food, as well as with harmful entomopathogens. To preserve their food source and secure their survival, *A. compressa* larvae can thus be expected to have evolved effective antimicrobial strategies, such as the deployment of antimicrobial compounds that inhibit growth of antagonistic bacteria and fungi.

In the present study, we screened *P. americana* hosts for the presence of possible pathogens using microbiological and molecular techniques. We extracted *P. americana* cockroaches parasitized by *A. compressa* and performed chemical analyses for structure elucidation and quantification of the compounds present in these extracts. We unraveled the source of the identified chemicals and, as a final step, examined their antimicrobial activity against an isolated pathogen and another bacterium in vitro.

Results

To detect microorganisms that may threaten *A. compressa* larvae during their development, we probed the outer surface and inner tissue of four *P. americana* cockroaches with microbiological techniques. From four of the surface samples and three of the tissue samples, we obtained bacterial colonies on agar plates. Nearly all of the colonies showed conspicuous pink pigmentation, and we focused on these pink colonies for further analyses. After purification of the bacterial strains, their DNA was extracted and partial 16S rRNA sequences were amplified by PCR and sequenced. The obtained partial 16S rRNA sequences

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. JX448402).

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of the four surface isolates (563, 586, 646, and 1,053 bp) and of the three tissue isolates (479, 492, and 510 bp) were all identical (GenBank accession no. of an exemplary 1,053 bp sequence: JX448402) and showed 99% homology with a published 16S rRNA gene sequence of *Serratia marcescens* (GenBank accession no. EF035134.1).

To elucidate the antimicrobial strategy that *A. compressa* larvae use, we monitored 8-d-old larvae inside their hosts through small holes in the abdominal cuticle covered by coverslips. The larvae could be observed to repeatedly deposit clear droplets of an oral secretion into the nearly empty body cavity of the cockroach, as well as onto the coverslips (Fig. 1 and Movie S1). The larvae then dispersed these droplets inside their hosts' bodies. Droplets of the secretion were directly probed from the coverslips by solid-phase microextraction (SPME) and analyzed by coupled gas chromatography–mass spectrometry (GC/MS). Apart from some typical insect hydrocarbons, the secretion contained nine more polar compounds that were neither previously known from *A. compressa* nor from *P. americana*. We therefore considered these compounds as candidate antimicrobial substances. The two major compounds of this mixture (1 and 8 in Table 1 and Fig. 2) were of particular interest.

To obtain larger amounts of the target compounds for structure elucidation and in vitro tests of antimicrobial activity, we extracted them with hexane from *P. americana* cockroaches parasitized by *A. compressa*. These extracts contained the target compounds in similar proportions as detected in the larval secretion (Fig. 2 and Table 1). Additionally, some typical cuticular hydrocarbons from *P. americana* and *A. compressa* were detected (Table S1). Control extracts of unparasitized *P. americana* contained only hydrocarbons (Fig. S1 and Table S1).

For structure elucidation, hexane extracts of parasitized cockroaches were purified by adsorption chromatography using a silica gel column and dichloromethane as the eluent, fractionated by size-exclusion high-performance liquid chromatography (SE-HPLC), and subsequently analyzed by GC/MS. The mass spectrum of major compound 1 (Fig. S2) exhibited a molecular ion at *m/z* 178 and matched mass spectral data published for the isocoumarin derivative mellein (16, 17). The optical rotation value of the purified compound 1 [$(\alpha_D) = -102°$ (CH_2Cl_2, $c = 0.05$)] was in agreement with that reported for (*R*)-(-)-mellein [(*R*)-(-)-3,4-diydro-8-hydroxy-3-methylisocoumarin] (18). To confirm the identity of compound 1, we synthesized (*R*)-(-)-mellein by published methods (19). Mass spectral data, retention time, and optical rotation of the synthesized (*R*)-(-)-mellein matched the data of the natural product. Thus, compound 1 was unambiguously identified as (*R*)-(-)-mellein. Apart from (*R*)-(-)-mellein, the oral secretion of *A. compressa* larvae contained some structurally related minor compounds, which were tentatively identified as hydroxylated mellein derivatives (Fig. 2 and Table 1).

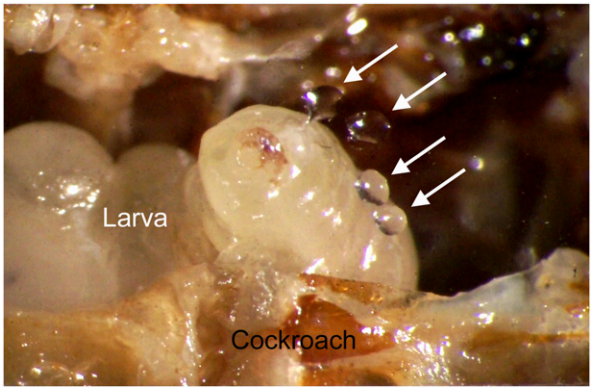

Fig. 1. A larva of *A. compressa* inside its *P. americana* host applying droplets of secretion (arrows) to a coverslip that covers an artificial opening in the cockroach cuticle (Movie S1).

The mass spectrum of compound 8 (Fig. S3) had a molecular ion at *m/z* 280 and matched mass spectral data reported for a monounsaturated C_{18}-γ-lactone (20–22). Derivatization of compound 8 with dimethyl disulfide (23) resulted in a product showing a molecular ion at *m/z* 374 and diagnostic ions at *m/z* 173 and 201 (Fig. S3), which is indicative of a double bond in position 9 (20). To elucidate the absolute configuration at carbon atom 4, we analyzed compound 8 and a racemic mixture of synthetic (9*Z*)-octadec-9-en-4-olide, as well as the pure (*S*)-enantiomer on an enantioselective β-DEX-225 GC column. Compound 8 coeluted with the earlier eluting (*R*)-enantiomer. To confirm the identity and absolute configuration of compound 8, we synthesized (4*R*,9*Z*)-octadec-9-en-4-olide according to refs. 24 and 25. The synthetic compound and the natural compound had identical mass spectra and retention times on both conventional and enantioselective GC columns. The synthesis of the (*Z*)-configured lactone yielded the (*E*)-configured isomer as a by-product that eluted slightly after the (*Z*)-configured isomer on the nonpolar GC column (20). Comparison with the natural product revealed that it contained only the (*Z*)-configured isomer. Therefore, the identity of compound 8 could be confirmed as (4*R*,9*Z*)-octadec-9-en-4-olide, also known under the common name micromolide (22), which will be used in the following text. Apart from micromolide, the oral secretion of *A. compressa* contained minor amounts of some additional long-chain γ-lactones (Fig. 2 and Table 1).

Quantitative analysis of single parasitized cockroaches (Table 1) revealed that (*R*)-(-)-mellein was by far the most prevalent compound, followed by micromolide. The total amount of the nine larval compounds per nest varied between 517 and 5,394 μg with a mean of 2,254 ± 1,869 μg.

As a next step, we tested our hypothesis that the oral secretion applied by *A. compressa* larvae to their cockroach hosts provides protection against antagonistic microbes. To this end, we tested the antibacterial activity of the dichloromethane fraction containing (*R*)-(-)-mellein and micromolide in broth microdilution assays against the isolated Gram-negative entomopathogen *S. marcescens* and against *Staphylococcus hyicus* as a selected Gram-positive bacterium. We determined the IC_{50}, i.e., the concentration of the antimicrobial compounds that is required to reduce bacterial growth by 50%, and we defined a "nest equivalent" as the average amount of antimicrobials found in one parasitized cockroach containing an *A. compressa* cocoon. The dichloromethane fraction effectively inhibited growth of *S. marcescens* (Fig. 3). There was a pronounced dose–response relationship with an IC_{50} of 0.18 nest equivalents/200 μL broth (*Materials and Methods* and *SI Materials and Methods*). A similar effect was found against *S. hyicus*, with an IC_{50} of 0.19 nest equivalents/200 μL. Thus, the fraction of the larval secretion containing (*R*)-(-)-mellein and micromolide clearly showed activity against both tested bacteria.

To elucidate the role of the two major compounds of the larval secretion separately, we tested synthetic (*R*)-(-)-mellein and micromolide in broth microdilution assays. (*R*)-(-)-mellein alone exhibited antibacterial activity against *S. marcescens* with an IC_{50} of 0.11 nest equivalents/200 μL. The highest concentration showed complete growth inhibition. Against *S. hyicus*, (*R*)-(-)-mellein was only mildly active with a growth inhibition of 66% at the highest concentration of about 0.7 nest equivalents/200 μL. Micromolide was hardly active against *S. marcescens* when tested alone; only the two highest concentrations affected its growth (inhibition of 16% and 26%, respectively). Against *S. hyicus*, micromolide was active with an IC_{50} value of 1.4 nest equivalents/200 μL. Concentrations of ≥2.7 nest equivalents/200 μL completely inhibited growth of *S. hyicus*. Both (*R*)-(-)-mellein and micromolide thus inhibited

Table 1. Chemical composition of the secretion of *A. compressa* larvae and mean amounts and respective proportions of larval compounds found on single *P. americana* hosts parasitized by *A. compressa* (n = 10)

No.	LRI	Compound	Larval secretion	Amount per cockroach (μg)	Proportion (%)
1	**1,550**	**(*R*)-(-)-mellein***	+	**1,900 ± 1,442**	**87 ± 5**
2	1,660	7-Hydroxymellein†	+	54 ± 110	1.3 ± 3.0
3	1,713	4-Hydroxymellein†	+	24 ± 21	1 ± 0.3
4	1,878	5-Hydroxymellein†	+	1.7 ± 5	0.04 ± 0.14
5	2,107	(*R*)-Hexadecan-4-olide‡	+	6.5 ± 7	0.22 ± 0.17
6	2,207	Heptadecan-4-olide§	+	0.6 ± 1.2	0.01 ± 0.03
7	2,273	Octadeca-9,12-dien-4-olide§	+	10 ± 9.2	0.46 ± 0.23
8	**2,284**	**(4*R*,9*Z*)-octadec-9-en-4-olide/micromolide‡**	+	**216 ± 205**	**8.6 ± 3.8**
9	2,313	(*R*)-octadecan-4-olide‡	+	41 ± 43	1.5 ± 0.8

The two major compounds are in boldface type. Numbers correspond to the numbers in Fig. 2. LRI, linear retention index (calculated in relation to *n*-alkanes); +, compound is present in larval secretion. Values are means ± SD.

*Identified by comparing the mass spectrum, retention time on a nonpolar GC column, and optical rotation value with a synthetic reference compound.

†Tentatively identified by comparing the mass spectrum with published mass spectra (*SI Materials and Methods*).

‡Identified by comparing the mass spectrum and retention times on a nonpolar and an enantioselective GC-column with a synthetic reference compound.

§Tentatively identified by interpretation of the mass spectrum (*SI Materials and Methods*).

bacterial growth in vitro but showed differential activity against the Gram-negative and Gram-positive target strains.

The antibacterial activity of (*R*)-(-)-mellein alone could explain the inhibitory effect of the fractionated secretion against the Gram-negative *S. marcescens* in our tests. However, neither (*R*)-(-)-mellein nor micromolide alone could explain the effect against the Gram-positive *S. hyicus*. We therefore performed an additional broth microdilution experiment to test whether the antimicrobial activity of the bioactive fraction was due to the combined activity of (*R*)-(-)-mellein and micromolide. A blend of the two synthetic compounds in the same ratio as found in the bioactive fraction showed the same activity as this fraction (median growth inhibition ± median absolute deviation: 91 ± 1.17% vs. 92 ± 1.17%; n = 6, U = 17, exact P = 0.9).

Discussion

As cockroaches are the exclusive hosts of *A. compressa* larvae, representing both their food source and microenvironment, the wasp larvae need effective defense mechanisms against competitive, putrefactive, and pathogenic microbes (8–13). In this study, we found clear evidence that *A. compressa* larvae are capable of coping with antagonistic microbes inside their *P. americana* hosts by using a mixture of antimicrobials present in their oral secretion.

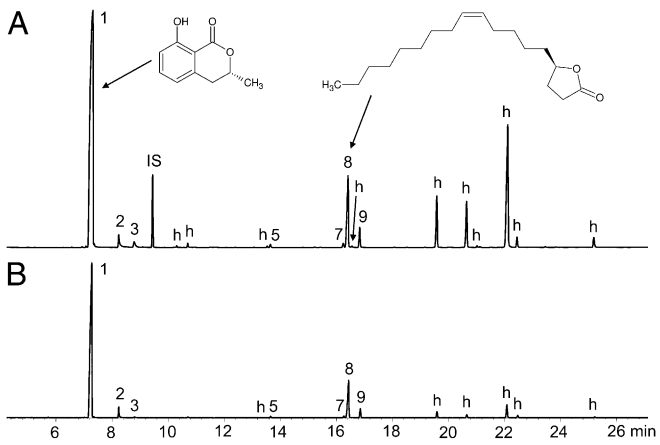

Fig. 2. Total ion current chromatograms of (*A*) a solvent extract of a *P. americana* parasitized by *A. compressa* and (*B*) a SPME sample of the secretion of an *A. compressa* larva. Although the relative amounts of compounds showed a broad variation, the chromatogram can be considered typical. The peaks of minor components are not always visible due to the magnification used. Peak numbers correspond to the numbers in Table 1. h, hydrocarbon; IS, internal standard; 1, (*R*)-(-)-mellein; 8, micromolide (Table S1 and Fig. S1).

We isolated the bacterium *S. marcescens* as the by far most abundant microbe from *P. americana* host cockroaches. *S. marcescens* (Enterobacteriaceae) is a ubiquitous, Gram-negative bacterium and a common colonizer of cockroaches (10–13). It is responsible for food contamination (26) and causes disease in plants (27), invertebrates (4, 26, 28), and vertebrates, including humans (29). Most important for this study, *S. marcescens* has been shown to cause severe septicemia in insect larvae, leading to their rapid death (4, 28, 30). *S. marcescens* thus may pose severe threats to the developing *A. compressa* offspring, which have to defend themselves against this virulent microbe.

Our behavioral observations and chemical analyses revealed that the larvae excreted and dispersed an oral secretion inside their cockroach hosts. This larval secretion contained the two major compounds (*R*)-(-)-mellein and micromolide, as well as some minor compounds. The same composition of compounds could be extracted from *P. americana* cockroaches parasitized by *A. compressa* in large quantities but not from unparasitized cockroaches. We thus conclude that the wasp larvae impregnate their cockroach hosts with these compounds from inside.

The dichloromethane fraction of the secretion containing (*R*)-(-)-mellein and micromolide showed antibacterial activity against *S. marcescens* and *S. hyicus*. Even though it is not known whether our chosen test strain, *S. hyicus*, is a natural target of the larval secretion, our assays demonstrate the potential of the larval secretion to inhibit growth of both Gram-negative as well as Gram-positive bacteria. These findings suggest that the wasp larvae sanitize their hosts with an antimicrobial secretion. The results of our assays revealed that the antimicrobial activity of the larval secretion is mediated by the two major compounds identified in this study. We could demonstrate that (*R*)-(-)-mellein effectively inhibits the growth of the Gram-negative *S. marcescens* and that it is mildly active against the Gram-positive *S. hyicus*. (*R*)-(-)-mellein has previously been isolated from diverse fungi (e.g., refs. 31 and 32), plants (33), and insects (34, 35). It has been shown to have antibacterial activity against standard and methicillin-resistant strains of *Staphylococcus aureus* (36). Moreover, it has antifungal activity (37, 38) and is a potent inhibitor of hepatitis C virus protease NS3 (39). However, to our knowledge, (*R*)-(-)-mellein has never been reported before as an antimicrobial agent used by an insect.

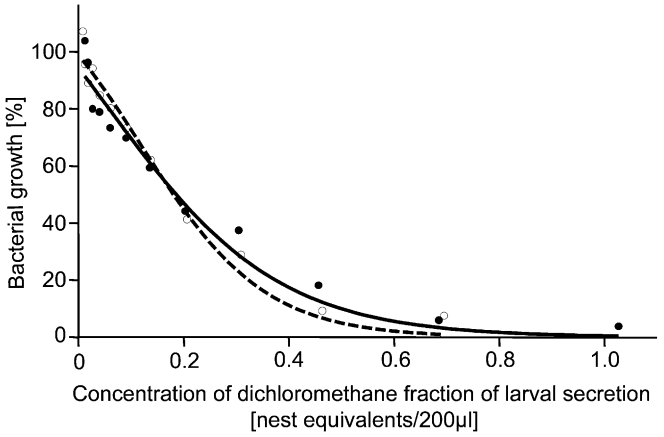

Fig. 3. Percentage of growth relative to controls of bacteria grown in the presence of varying concentrations of the dichloromethane fraction of the larval secretion. Growth was measured as optical density in broth microdilution assays. Logistic regressions with Levenberg–Marquardt correction were fitted to the data. Open circles and dashed line: *S. marcescens*; filled circles and solid line: *S. hyicus*.

The second major compound of the larval secretion, micromolide, displayed clear growth-inhibiting properties against *S. hyicus*, but not against *S. marcescens*. Micromolide, which has previously been isolated as the sex pheromone of the currant stem girdler *Janus integer* [Hymenoptera, Cephidae (20, 21)] and from the bark of *Micromelum hirsutum* [Sapindales, Rutaceae (22)], is one of the most active compounds against the causative agent of tuberculosis, *Mycobacterium tuberculosis* and is thought to be a promising lead compound for the development of new antituberculosis drugs (22).

The combination of (*R*)-(-)-mellein and micromolide, as found in the secretion of *A. compressa* larvae, has so far not been reported from any natural source. As this blend of compounds can be seen as the result of an evolutionary arms race between the wasp and pathogenic microorganisms over long periods of time, one can expect that the use of this particular mixture lends a decisive advantage to the wasp larvae with regard to survival and/or well-being. Our antimicrobial assays revealed that the effect of the bioactive fraction against *S. marcescens* could be explained by the activity of (*R*)-(-)-mellein alone. Against *S. hyicus*, the synthetic blend of micromolide and (*R*)-(-)-mellein was similarly active in our assays compared with the bioactive fraction. This suggests that the inhibitory effect of the fraction against *S. hyicus* is mediated by the combined activity of micromolide and (*R*)-(-)-mellein. Hence, our own results and previous reports (22, 36–39) suggest that the specific combination of antimicrobials present in the larval secretion of *A. compressa* exert activity against a wide range of different microbes.

Such a broad-spectrum defense may be indispensable to the life of *A. compressa* larvae. The range of microbes that *A. compressa* larvae may encounter during their development in their hosts is unpredictable and may encompass all different kinds of microbes, such as various Gram-positive and Gram-negative bacteria, mycobacteria, viruses, yeasts, and filamentous fungi (8, 9, 11, 12, 14, 40). An effective strategy to gain a reliable and enduring protection against a broad spectrum of microbes may therefore be the application of large amounts of a mixture of several relatively unspecific antimicrobial compounds (41, 42).

The focus of the present study was to investigate the major compounds of the larval secretion. The methods used for extraction and fractionation were optimized to achieve this goal. Hence, the crude larval secretion might contain additional polar compounds that contribute to its antimicrobial property but that were not covered by our analytical approach. However, taking into account that the concentrations of (*R*)-(-)-mellein and micromolide in the antimicrobial assays are similar to those found in the cockroaches (*SI Materials and Methods*), the IC_{50} values determined here for *S. marcescens* and *S. hyicus* indicate that the average amount of antimicrobials released by a single larva is ample to effectively impair bacterial growth in its cockroach host. The large amounts of antimicrobials deployed suggest that *A. compressa* larvae invest a considerable portion of their resources into their antimicrobial defense.

Given that virtually all insect species are threatened by pathogenic and competing microorganisms, it can be assumed that all species have to use some kind of countermeasure. Recently, such antimicrobial defense mechanisms of insects have received growing attention, and their potential as valuable sources of natural products and therapies has been acknowledged (43–47).

Food hygiene may be of vital importance, especially to the vulnerable early developmental stages of insects to evade the threats of antagonistic microbes. Even though the antimicrobial strategies used to protect the immatures of insects from food-associated microbes remain rather unexplored, there are some examples that demonstrate the diversity and ingenuity of remedial measures that insect larvae or their parents use against food-associated microbes. Larvae of the parasitoid ichneumonoid wasp *Pimpla turionellae*, for example, discharge an antimicrobial anal secretion of unknown composition inside their lepidopteran host pupae to secure their survival (48). Females of the parasitoid wasp *Philanthus triangulum* embalm their prey bees with a lipid secretion that reduces water condensation and in this way inhibit the growth of fungal decomposers on the larval provisions (49). Adult burying beetles of the genus *Nicrophorus* use antimicrobial oral and anal exudates to protect the carrion that serves as larval food from microbes (46, 50).

We finally conclude that the impregnation of the host with large amounts of antimicrobial substances is a (food-) hygienic behavior of the *A. compressa* larva that grants protection against antagonistic microbes during development inside the cockroach host. The secretion of a blend of antimicrobials with broad-spectrum activity seems to represent an essential frontline defense strategy against diverse putrefactive and entomopathogenic microbes.

Materials and Methods

Insects. *P. americana* parasitized by *A. compressa* were obtained from laboratory populations kept at the University of Regensburg (*SI Materials and Methods*).

Isolation and Identification of Microorganisms from Cockroaches. The outer surfaces of four *P. americana* were probed with nylon-flocked swabs (microRheologics), which were then streaked onto lysogeny broth (LB) agar plates. Four additional *P. americana* cockroaches were surface-sterilized with 70% (vol/vol) ethanol and dissected with sterile dissecting scissors and forceps to remove thoracic muscle tissue, as this is the tissue that *A. compressa* larvae feed on. The muscle tissue was then streaked onto LB agar plates. The plates were incubated at 30 °C until visible growth of microbes. Morphological characteristics of the cultivated strains were recorded. Single bacterial colonies were transferred twice to new LB agar plates to obtain pure strains. Genetic analyses were performed as described in *SI Materials and Methods*.

Behavioral Observations. To study the behavior of *A. compressa* larvae inside their hosts, parasitized *P. americana* ($n = 7$) were dissected 8 d after oviposition by the *A. compressa* female. The abdominal cavity of the cockroach was opened by carefully removing the right half of each sternite. The hole in the cockroach carcass was covered by a glass coverslip, which allowed the observation of the larva. For documentation of the behavior, high-definition movies and photos were taken with a Sony NEX-5 digital camera mounted on a Zeiss OPMI stereomicroscope.

A. compressa larvae could be observed excreting small droplets of liquid onto the coverslip (*Results*). These droplets of larval secretion were taken up with a SPME fiber (100 µm polydimethylsiloxane; Supelco) by wiping the

fiber in the droplets. The fiber was then allowed to air dry for 1 min and subsequently analyzed by coupled GC/MS (*SI Materials and Methods*).

Isolation and Fractionation of Chemical Compounds. Preliminary GC/MS analyses of extracts made with hexane, dichloromethane, and methanol revealed no marked differences. Given that compounds 1 and 8 were by far the most prevalent compounds in each of the extracts, we focused on the isolation and identification of these two major compounds (and the structurally related minor compounds) to test our hypothesis of their antibacterial activity.

For identification of putative antimicrobials, five parasitized cockroaches containing *A. compressa* cocoons were extracted in 10 mL hexane for 10 min. The crude hexane extract was then fractionated by column chromatography on a silica gel column (Chromabond 500 mg; Macherey and Nagel) with hexane and dichloromethane (8 mL each) as the mobile phase to separate nonpolar hydrocarbons from the more polar target compounds. GC/MS analyses revealed that the hexane fraction contained only hydrocarbons, whereas the dichloromethane fraction contained the more polar target compounds. Using dichloromethane as the mobile phase, the dichloromethane fraction of the extract was further fractionated by SE-HPLC (*SI Materials and Methods*) to separate (*R*)-(-)-mellein and the minor isocoumarine derivatives from micromolide and the minor γ-lactones. Two fractions containing the γ-lactones (fraction 1, retention time 6.35–7.20 min) and the isocoumarins (fraction 2, 7.34–8.04 min), respectively, were collected manually.

Identification of Chemical Compounds. Identification of the putatively antimicrobial compounds was accomplished by use of GC/MS, enantioselective gas chromatography, and determination of optical rotation (*SI Materials and Methods*). Iodine-catalyzed methylthiolation using dimethyl disulfide (23) was performed to determine the positions of the double bonds of unsaturated compounds.

(*R*)-(-)-mellein was synthesized according to ref. 19, and its purity was confirmed by comparing the NMR spectrum, melting point, and optical rotation value with data previously reported (18). Micromolide was synthesized according to refs. 24 and 25, and its purity was confirmed by comparing the NMR spectrum, melting point, and optical rotation value with data previously reported (51). A racemic mixture of (9*Z*)-octadec-9-en-4-olide and enantiomerically pure (4*S*,9*Z*)-octadec-9-en-4-olide were kindly provided by Allard Cossé, Agricultural Research Service, Department of Agriculture, Preoria, IL. Racemic mixtures of hexadecan-4-olide and octadecan-4-olide were kindly provided by Lukas J. Goossen and Dominik M. Ohlmann, Fachbereich Chemie, Technische Universität Kaiserslautern, Germany (52).

(*R*)-(-)-mellein was identified in the natural sample by comparing the mass spectrum, retention time on a nonpolar GC column, and optical rotation value with data obtained by analysis of the synthetic reference compound. The optical rotation values were measured with a Perkin-Elmer 241 polarimeter ($c = 0.05$ in dichloromethane). Micromolide and the two minor lactones, hexadecan-4-olide and octadecan-4-olide, were identified by comparing their mass spectra and retention times on both nonpolar and enantioselective GC columns with data obtained by the analyses of the synthetic reference compounds. For information on the minor compounds, see *SI Materials and Methods*. To confirm the absolute configuration of the γ-lactones 8 (micromolide), 5, and 9, we performed enantioselective gas chromatography on a β-DEX 225 stationary phase (*SI Materials and Methods*). Hydrocarbons in the nonpolar fraction were identified as previously described (53).

Quantification of Chemical Compounds. For quantification of the antimicrobials found on single parasitized cockroaches, 10 cockroaches containing *A. compressa* cocoons were individually extracted in 4 mL of dichloromethane containing 0.05 mg·mL^{-1} octadecane as an internal standard under gentle agitation for 2 h. As controls, eight unparasitized cockroaches were extracted in the same way. An aliquot of 1 μL of each sample was used for GC/MS analysis. Quantification of (*R*)-(-)-mellein and micromolide in individual extracts was done by the internal standard method. For this purpose, calibration curves were created by analyses of a dilution series of synthetic (*R*)-(-)-mellein and micromolide dissolved in dichloromethane containing the internal standard. Values given are means ± 1 SD.

In Vitro Tests of Antimicrobial Activity: Broth Microdilution Assays. The antibacterial activity of the dichloromethane fraction of the larval secretion containing (*R*)-(-)-mellein and micromolide was investigated with broth microdilution assays (*SI Materials and Methods*) against the target strains *S. marcescens* (isolated in the present study) and *S. hyicus* (obtained from the Institute of Microbiology at the University of Regensburg). *S. marcescens* was used because it was isolated from *P. americana* in this study and represents a potential threat to *A. compressa* immatures. *S. hyicus* was chosen to test the activity of the dichloromethane fraction of the larval secretion against a representative of the Gram-positive bacteria. Synthetic samples of (*R*)-(-)-mellein and micromolide were assayed individually for their antibacterial activity.

The data were visualized as concentration vs. growth response curves. Nonlinear regression was performed by fitting logistic regression lines with a Levenberg–Marquardt correction to the data set using the statistics software package PAST, Version 2.5 (54). The IC_{50} was calculated by using the regression equations.

To verify that the antibacterial activity of the dichloromethane fraction was mediated by (*R*)-(-)-mellein and micromolide and not by an unknown nonvolatile component that was not detectable in our GC/MS analyses, we tested the antimicrobial activity of a synthetic blend of (*R*)-(-)-mellein and micromolide in the same ratio as found in the bioactive dichloromethane fraction against this fraction in broth microdilution experiments against *S. hyicus* (*SI Materials and Methods*). The median growth inhibition of the fraction and the synthetic blend was compared with an exact Mann–Whitney *U* test.

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