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Sensitization of *Staphylococcus aureus* and *Escherichia coli* to Antibiotics by the Sesquiterpenoids Nerolidol, Farnesol, Bisabolol, and Apritone

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The sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone were investigated for their abilities to enhance bacterial permeability and susceptibility to exogenous antimicrobial compounds. Initially, it was observed by flow cytometry that these sesquiterpenoids promoted the intracellular accumulation of the membrane-impermeant nucleic acid stain ethidium bromide by live cells of *Lactobacillus fermentum*, suggesting that enhanced permeability resulted from disruption of the cytoplasmic membrane. The ability of these sesquiterpenoids to increase bacterial susceptibility to a number of clinically important antibiotics was then investigated. In disk diffusion assays, treatment with low concentrations (0.5 to 2 mM) of nerolidol, bisabolol, or apritone enhanced the susceptibility of *Staphylococcus aureus* to ciprofloxacin, clindamycin, erythromycin, gentamicin, tetracycline, and vancomycin. Nerolidol and farnesol also sensitized *Escherichia coli* to polymyxin B. Our results indicate the practical utility of sensitizing bacteria to antimicrobials with sesquiterpenoids that have traditionally been used as flavorants and aroma compounds in the food and perfume industries.

Increasing bacterial resistance to antibiotics and antimicrobials is a growing concern facing the medical, food, and sanitation industries (8, 11). Major mechanisms of bacterial resistance to antimicrobials include active drug efflux systems, mutations that result in altered cell permeability, cellular degradation of antimicrobials, and alterations of their cellular targets (14, 17). In efforts to counter the increasing incidence of antibiotic resistance, the pharmaceutical and food industries have invested substantial resources in the search for new inhibitory compounds of microbial, plant, and animal origin. Additionally, new synthetic and semisynthetic antibiotics that are resistant to enzymatic degradation or modification have been introduced (14, 17). Although specific mechanisms of bacterial resistance to antimicrobials have been characterized and bypassed in certain cases, the more general mechanisms of altered cellular permeability to antimicrobial agents are much less understood on a physical and molecular basis. Addressing cellular permeability barriers by using methods for enhancing the uptake of antimicrobial agents could therefore represent an important means both for decreasing antibiotic usage levels and in preventing resistance, as alterations in permeability would be expected to involve a number of genes and require modification of the structurally complex cell membrane and cell wall structures of microorganisms.

Terpenoids are a broad class of lipophilic secondary metabolites derived from mevalonate and isopentenyl pyrophosphate and occur widely in nature (6, 7, 13). The greatest diversity of terpenoid structures occurs in plants, and collectively they form a major component of the "essential oil" fraction of plant extracts. Sesquiterpenoid compounds, containing 15 carbons, are formed biosynthetically from three five-carbon isoprene units or are synthesized industrially from monoterpenoid feedstocks (1). These compounds have been of industrial interest primarily for their contribution to the characteristic flavors and aromas of herbs, spices, and flowers (1). Apart from these uses, sesquiterpenoids have been associated with a variety of important biological functions such as pheromones, insect antifeedants, or phytoalexins (6). Recently, quorum sensing activities have been described for the sesquiterpenoids farnesol and nerolidol. In this role, these compounds prevent the transition from yeast-like to mycelial growth in *Candida albicans* (9). Because pathogenicity is associated with mycelial forms of *C. albicans*, suppression of the yeast-to-mycelial transition may represent a novel means for nonlethal control of this opportunistic pathogen (9).

Intriguing work has been done using both monoterpenoids and sesquiterpenoids as skin penetration enhancers for the transdermal delivery of therapeutic drugs. In this application, these compounds are used either as undiluted oils or as minimally diluted solutions in small amounts of solvents such as propylene glycol (2, 3). Various plant essential oils have also been investigated for use as food-grade antimicrobials, but to be effective, they are generally applied at high levels that are often impractical (5).

In this study, we have found that low (millimolar) concentrations of the sesquiterpenoid flavorant and aroma compounds nerolidol, farnesol, bisabolol, and apritone can nonspecifically enhance the permeability of bacterial cells to certain exogenous chemical compounds, including antimicrobial agents. We have applied this finding as a means of enhancing bacterial susceptibility to antibiotics and other antimicrobial compounds.

Sesquiterpenoid-mediated uptake of ethidium bromide by

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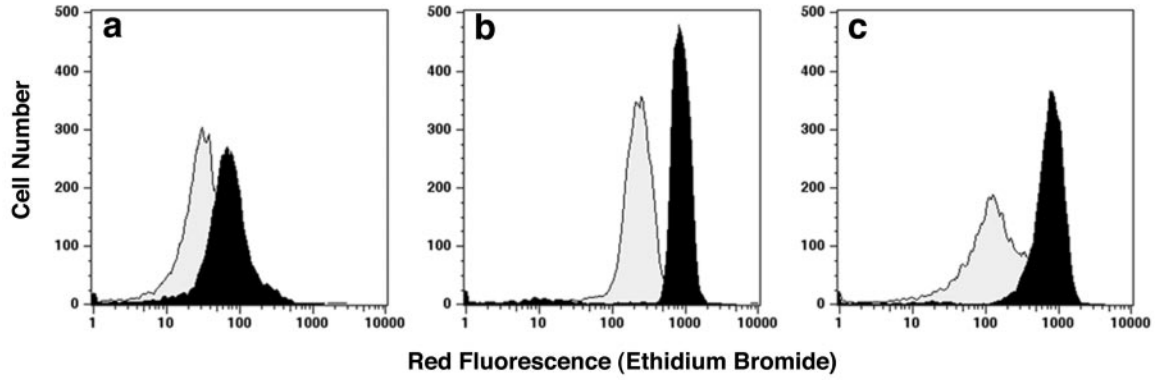

FIG. 1. Ethidium bromide accumulation by sesquiterpenoid-treated cells of *L. fermentum*. Cells treated with nerolidol (0.5 mM, black histograms) or ethanol (0.5%, gray histograms) were examined immediately after addition of ethidium bromide (a), 5 min later (b), and after 40 min of exposure to ethidium bromide (c).

***Lactobacillus fermentum*.** Unless noted otherwise, all chemicals used in this study were from Sigma (St. Louis, Mo.). To initially determine the ability of sesquiterpenoids to permeabilize gram-positive bacterial cells, we used a flow cytometric assay for the uptake of ethidium bromide by *L. fermentum* (ATCC 14931). This nonpathogenic organism was chosen for reasons of safety as a model gram-positive bacterium for live cell cytometry experiments. Ethidium bromide (FW 394.3) is a membrane-impermeant model drug with limited intrinsic fluorescence. When cells with damaged or compromised cell membranes are treated with this probe, it enters the cell, where it intercalates within double-stranded DNA and becomes highly fluorescent. Because of these properties, ethidium bromide can be a useful reporter of membrane integrity (12).

An overnight (18- to 20-h) culture of *L. fermentum* grown at 30°C in MRS broth (Difco) was diluted to a concentration of 10^7 CFU ml^{-1} in neutral phosphate buffer (5.83 g of NaH_2PO_4 and 6.74 g of Na_2HPO_4 per liter of distilled water; pH 7.0). Cell suspensions were then treated with 0.5 mM sesquiterpenoid (in ethanol, 0.5% final concentration) and 15 μM ethidium bromide and incubated for up to 40 min at 25°C. After incubation, cell suspensions were diluted 1:10 in the same buffer and examined using a FACScan flow cytometer (BD Biosciences, San Jose, Calif.). Cells heated for 6 min in a 55°C water bath with constant shaking served as a positive control for ethidium bromide uptake. As assessed by flow cytometry, cells of *L. fermentum* treated with 0.5 mM nerolidol, farnesol, bisabolol, or apritone and stained for up to 40 min with ethidium bromide were more fluorescent and more homogeneously stained than cells treated with 0.5% ethanol and ethidium bromide alone. Figure 1 illustrates these results for nerolidol-treated cells. Sesquiterpenoid-mediated enhancement of ethidium bromide uptake was seen immediately upon addition of 0.5 mM nerolidol (Fig. 1a). After 5 min, the nerolidol-treated population was markedly brighter and more uniformly stained than the control population (Fig. 1b). After 40 min of exposure, the ethanol-treated control population was more heterogeneously stained with ethidium bromide, while the nerolidol-treated population remained brighter and more uniformly fluorescent (Fig. 1c).

Enhanced susceptibility of *Staphylococcus aureus* to antibiotics. Because the flow cytometry experiments showed that sesquiterpenoids could effectively permeabilize *L. fermentum* to ethidium bromide, we next examined the more general ability of these compounds to enhance bacterial susceptibility to other low-molecular-weight compounds, particularly antibiotics, including ciprofloxacin, clindamycin, erythromycin, gentamicin, tetracycline, and vancomycin. The ability of sesquiterpenoids to sensitize *S. aureus* (ATCC 6538) to antibiotics was assessed using a disk diffusion assay. Cells were grown overnight (18 to 20 h) at 30°C in 10 ml of Trypticase soy broth (Becton Dickinson and Company, Franklin Lakes, N.J.) and diluted to a concentration of 10^7 CFU ml^{-1} in neutral phosphate buffer. An 0.5-ml aliquot of this suspension was combined with 4.5 ml of an overlay agar prepared from Iso-Sensitest broth (Oxoid, Ogdensburg, N.Y.) plus 0.7% agar, tempered to 50°C. Sesquiterpenoids (in ethanol, 0.5% final concentration) were added to the cell-overlay mixture, and the mixture was vortexed. After vortexing, treated cell overlays were poured over hardened Iso-Sensitest agar plates (2% agar) and the plates were swirled thoroughly in alternating clockwise and counterclockwise motions to help ensure an even distribution of sesquiterpenoids throughout the overlay. This was especially important with bisabolol. Without thorough mixing, bisabolol treatments often yielded asymmetric zones of inhibition. Antibiotic disks (BBL Sensi-Disc; Becton Dickinson and Company), were placed on hardened agar overlays, and plates were incubated at 37°C for 22 to 24 h. Diameters of zones of inhibition were measured with a ruler from the bottom of each plate. Here, as elsewhere in this work, experiments were repeated at least three times. However, absolute zone size for given antibiotic-sesquiterpenoid combinations varied slightly between independent experiments, likely as a result of limited miscibility of sesquiterpenoids in the agar overlay. Therefore, the values reported in Table 1 represent averages of duplicate plates for one experiment but accurately depict the trends seen in sesquiterpenoid enhancement activity. For photography (Fig. 2), cell suspensions were combined with tempered Iso-Sensitest agar as above and 2.5 ml of the resulting mixture was overlaid onto hardened Iso-Sensitest agar plates in 60-mm-diameter petri dishes.

Nerolidol, bisabolol, and apritone enhanced the activities of

TABLE 1. Antimicrobial activity observed by disk diffusion assay[a]

Antibiotic	Diam of zone of inhibition (mm)						
	Control	1 mM A	2 mM A	1 mM B	2 mM B	1 mM N	2 mM N
Erythromycin	24	26	26.5	27.5	29	29.5	33
Gentamicin	18.5	21	23	25	27	25.5	35
Vancomycin	18	18	20	19	21	22	25

[a] The effects of increasing sesquiterpenoid concentrations on the susceptibility of *S. aureus* to erythromycin (15 μg), gentamicin (10 μg), or vancomycin (30 μg) were assayed. Abbreviations: A, apritone; B, bisabolol; N, nerolidol.

all six antibiotics tested against *S. aureus*, with gentamicin showing the largest relative increases in inhibition zone size for each sesquiterpenoid tested. Antibiotic enhancement was apparent at sesquiterpenoid concentrations as low as 0.5 mM (data not shown) and increased correspondingly with sesquiterpenoid concentration. Nerolidol was the most effective antibiotic enhancer for *S. aureus*, followed by bisabolol and apritone. Because farnesol alone was inhibitory to this organism at the levels used (1 mM and above), its efficacy was not compared against those of the other sesquiterpenoids. Table 1 summarizes the inhibition zones observed with 1 or 2 mM apritone, bisabolol, or nerolidol together with erythromycin, gentamicin, or vancomycin against *S. aureus*. Zones of inhibition against *S. aureus* by ciprofloxacin, clindamycin, and tetracycline had diffuse edges and could not be accurately measured. Figure 2 illustrates the effects of 1 or 2 mM nerolidol against *S. aureus* with these antibiotics.

Enhanced susceptibility of *Escherichia coli* to polymyxin B. We also examined the enhancing activity of sesquiterpenoids with antibiotics against *E. coli* (ATCC 25922), which as a gram-negative bacterium possesses the additional permeability barrier of an outer membrane (OM) structure. The effect of nerolidol or farnesol on the antimicrobial activity of polymyxin B against *E. coli* was determined at various intervals by pour plating. Cells were grown and diluted in phosphate buffer as described above for *S. aureus* and treated with a 1 mM concentration of either sesquiterpenoid and/or 10 μg of polymyxin B sulfate (Sigma; 8,100 units mg^{-1}) ml^{-1}. Cell suspensions were incubated in a 37°C water bath for up to 30 min, and samples were removed at appropriate intervals, diluted in the same buffer, and enumerated by pour plating with Trypticase soy agar tempered to 50°C. Plates were incubated for 24 h at 37°C prior to counting of colonies. Cells of *E. coli* treated with sesquiterpenoid and polymyxin B were inactivated more rapidly than were cells treated with polymyxin alone. Figure 3 illustrates this enhancing activity for nerolidol. Under the conditions of this assay, sesquiterpenoids alone had no effect on the viability of this organism.

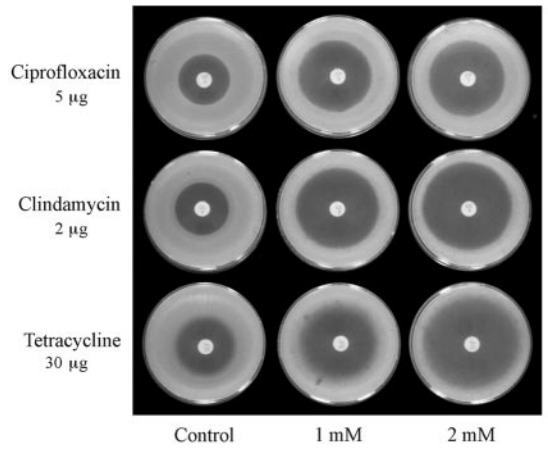

FIG. 2. Illustration of antimicrobial activity observed in the disk diffusion assay. The figure shows the enhanced antimicrobial activity effect of increasing concentrations of nerolidol on susceptibility of *S. aureus* to ciprofloxacin (5 μg), clindamycin (2 μg), or tetracycline (30 μg).

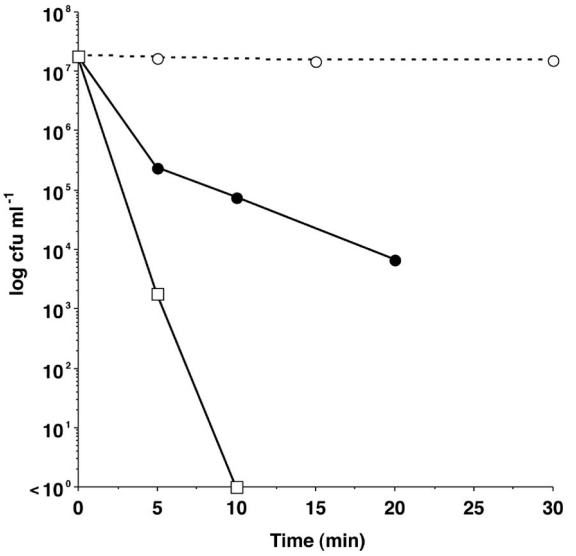

FIG. 3. Synergistic effect of nerolidol and polymyxin B against *E. coli* incubated at 37°C in neutral phosphate buffer. Symbols: ○, 1 mM nerolidol; ●, 10 μg of polymyxin B ml^{-1}; □, 1 mM nerolidol plus 10 μg of polymyxin B ml^{-1}.

Due to their inherent lipophilicities, terpenoid compounds show an affinity for and partition within biological membranes, where their accumulation may impact substantially on the structural and functional properties of these membranes (15). Our results indicate that the sesquiterpenoid compounds nerolidol, farnesol, bisabolol, and apritone can disrupt the normal barrier function of the bacterial cell membrane, allowing the permeation into the cell of exogenous solutes such as ethidium bromide and antibiotics. This effect is more pronounced for gram-positive bacteria, probably due to the lack of additional permeability barriers, particularly the OM of gram-negative bacteria. Still, the polymyxin results observed in this study suggest that sesquiterpenoids may also find applications in sensitizing gram-negative bacteria to other antibiotics or antimicrobials, provided that steps are taken to enhance the permeability of the OM or that antibacterial agents already possessing some OM-permeabilizing activities are used. Thus, it would be of interest to evaluate the sensitizing activities of these sesquiterpenoids in combination with compounds known to disrupt the OM (16).

In preliminary work, we also screened a number of cyclic and acyclic monoterpenoids for their abilities to enhance the susceptibility of *S. aureus* to the same antibiotics tested with the

sesquiterpenoids. However, these compounds demonstrated little, if any, effect as enhancing agents (data not shown). The increased effectiveness of sesquiterpenoids as enhancers of membrane permeability may stem from their structural resemblance to membrane lipids (e.g., linear molecules with internal lipophilic character and a more polar terminus). Cornwell and Barry (2) found nerolidol and farnesol to be more effective as skin penetration enhancers than bisabolol and proposed that the longer hydrocarbon tails of these molecules play an important functional role in promoting greater interaction with the interior of the bilayer than that promoted by other classes of terpenoids.

The antibiotics used against *S. aureus* in this study were selected on the basis of their clinical importance. Other than having primary activities against cytoplasmic targets, they have no unifying themes of structure or specific action. The antibiotics range in molecular weight from 331.3 (ciprofloxacin) to 1,449.2 (vancomycin), and they vary in character from lipophilic (erythromycin) to hydrophilic (gentamicin). The ability of sesquiterpenoid treatment to sensitize bacterial cells to such a heterogeneous group of antibiotics underlines the nonspecific and potentially general nature of this enhancing activity.

We expect that other classes of cytoplasmically targeted antimicrobial agents including food preservatives, biocides, and sanitizers as well as other antibiotics will also show enhanced antimicrobial activities in the presence of these sesquiterpenoids. However, because the principal interactions of terpenoids are with the cytoplasmic membrane, it is likely that the effects of membrane-active antimicrobial compounds will be especially enhanced. The results reported here for gentamicin and polymyxin B support this theory. In addition to its interaction with bacterial ribosomes, the cationic aminoglycoside gentamicin has also been shown to have membrane-destabilizing properties (10), and polymyxin B is well known for its actions against both the outer and cytoplasmic membranes (4). Additional work in this laboratory has shown that 2 mM nerolidol dramatically enhances the susceptibility of *Listeria monocytogenes* to a variety of membrane-targeted antimicrobials, including monolaurin, hop β-acids, and sodium desoxycholate (B. F. Brehm-Stecher and E. A. Johnson, unpublished results).

Our results suggest a general role for the use of sesquiterpenoid compounds as enhancers of nonspecific bacterial permeability to antibiotics and antimicrobials. In this capacity, these compounds may be useful to the medical, food, and sanitation industries, especially in topical or surface applications, where the required concentrations of sesquiterpenoids could reasonably be achieved. Potential applications include the treatment or prevention of infections, enhancement of food safety, and the fortification of existing sanitizing procedures. Since nerolidol, farnesol, and certain other sesquiterpenoids are generally recognized as safe (GRAS), these compounds may have special applications in concert with antimicrobials intended for use in foods or on food contact surfaces.

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