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Structural Requirements for the Antimicrobial Activity of Carvacrol

EDWIN J. A. VELDHUIZEN,*,†,‡ JOHANNA L. M. TJEERDSMA-VAN BOKHOVEN,†,‡ CINDY ZWEIJTZER,† SARA A. BURT,† AND HENK P. HAAGSMAN†,‡

Institute of Risk Assessment Sciences, Division of Public Health & Food Safety, and Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3508 TD Utrecht, The Netherlands

Carvacrol is a component of several essential oils and has been shown to exert antimicrobial activity. The structural requirements for the activity of carvacrol were determined by comparison to structurally related (nonessential oil) compounds. Removal of the aliphatic ring substituents of carvacrol slightly decreased the antimicrobial activity. The effect of the hydroxyl group of carvacrol on activity could not be determined by simply comparing it to *p*-cymene, because this compound is immiscible with water; therefore, 2-amino-*p*-cymene, the amino analogue of carvacrol, which has a similar hydrophobicity and structural characteristics, was used. 2-Amino-*p*-cymene had similar membrane disruption and bacterial killing characteristics as carvacrol showing that, contrary to previous reports, the hydroxyl group of carvacrol itself is not essential for the antimicrobial activity. However, the observed 3-fold lower activity for 2-amino-*p*-cymene as compared to carvacrol indicates special features in the antimicrobial mode of action of carvacrol due to the hydroxyl group.

KEYWORDS: Carvacrol; essential oil; antimicrobial activity

INTRODUCTION

The antimicrobial activity of essential oils (EOs) and their components has long been recognized, and interest has increased in recent years. In the food industry, there is a serious problem about the growing number of food borne illness outbreaks. EOs have promising potential as new natural food preservatives that could also meet Western society's desire for green products (*1*). Carvacrol, a phenolic compound, is considered one of the main components of certain EOs that exerts antimicrobial activity, not only because of its high abundance in some oils, which can reach levels of 75% (*2*, *3*), but also because of its high specific activity as compared to other EO components (*4*, *5*). Studies that have been performed on the antimicrobial activity of carvacrol have shown that it has a broad spectrum of antimicrobial activity against almost every Gram-positive and Gram-negative bacteria tested (*5*). Besides this antibacterial activity, carvacrol has been described as antifungal (*6*, *7*), antitoxigenic (*8*), insecticidal (*9*, *10*), and antiparasitic (*11*). Antiviral activity has been observed for whole EOs containing carvacrol as the major component (*12*, *13*), but only a very low antiviral activity has been observed for carvacrol alone (*3*).

Despite extensive research on carvacrol in recent years, not much is known about the mechanism of action of carvacrol against bacteria. The hydrophobicity of the compound indicates that the bacterial membrane is likely to be the initial target of the compound. In addition, carvacrol has previously been shown to disrupt the bacterial membrane, affect the proton motive force, and disrupt both the pH gradient and the electron flow across the membrane (*14*, *15*). Some studies on the structural requirements of carvacrol for its antimicrobial activity have been performed, mainly by comparing it to related EO components (*4*, *5*, *15*). In these studies, it has been shown that the two main characteristics of carvacrol are the hydroxyl group and the delocalized electrons of the benzene ring. However, because of the limited number of EO components, these comparisons are often hampered by the introduction of two or more structural changes in the compound. For example, the methyl ether of carvacrol (2-methoxy-4-isopropyl-1-methylbenzene) has been used to show the necessity of the hydroxyl group of carvacrol for antimicrobial activity (*4*, *15*). However, the elimination of the hydroxyl group by replacing it with the methyl ether affects the hydrophobicity of the molecule and the size of the ring substituent, both of which may have a major influence on specific interactions with bacterial cells.

The aim of this study is to determine the structural requirements for the antimicrobial activity of carvacrol. For this purpose, we use a methodological approach to compare carvacrol with non-EO compounds that would better suit a true comparison as compared to EO compounds. The structures of these compounds are shown in **Figure 1**. The compounds in this figure can be divided into two groups: In the first group, every substituent of the carvacrol benzene ring is deleted

* To whom correspondence should be addressed. Tel: +31 30 2535361. Fax: +31 30 2532365. E-mail: E.Veldhuizen@vet.uu.nl.
† Division of Public Health & Food Safety.
‡ Faculty of Veterinary Medicine.

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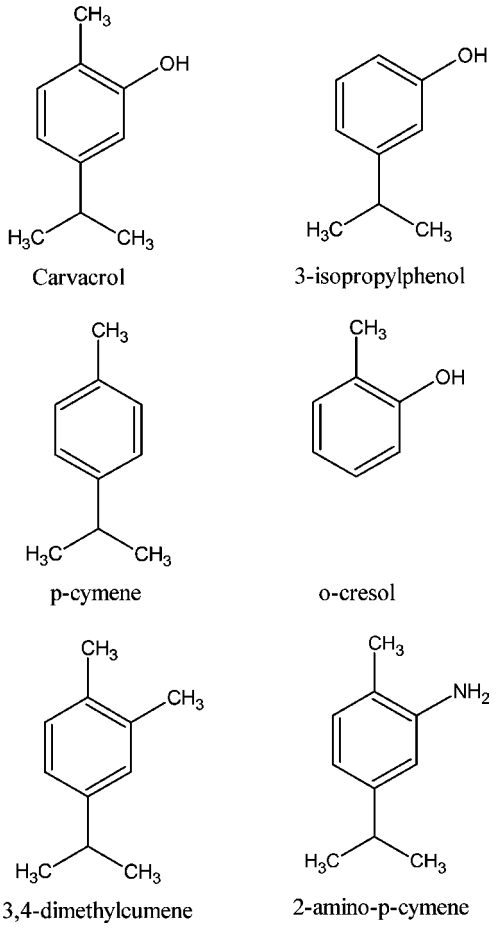

Figure 1. Structure of carvacrol and carvacrol-related compounds used in this study.

separately. This group contains 3-isopropylphenol, *o*-cresol, and *p*-cymene. The second group contains 2-amino-*p*-cymene and 3,4-dimethylcumene, compounds possessing amino and methyl groups, respectively, instead of the hydroxyl group in carvacrol.

MATERIALS AND METHODS

Chemicals. N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), carvacrol, 3-isopropylphenol, 2-amino-*p*-cymene, 3,4-dimethylcumene, *o*-cresol, and *p*-cymene were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 3,3′-Dipropylthiacarbocyanide iodide ($DiSC_3$5) was obtained from Molecular Probes. Valinomycin was obtained from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were of analytical grade.

Preparation of Cultures. Overnight cultures of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 6538) were prepared freshly for every experiment by cultivation from frozen stock at 37 °C for 16 h in Luria−Bertani (LB) broth. Before use in the antimicrobial assays described below, the optical density (OD) of the suspension was measured using a Pharmacia Ultrospec III spectrophotometer at 620 nm, and the suspensions were diluted in LB to the appropriate bacterial density needed in the experiments.

Determination of Compound Solubility. For determination of the solubility in LB of the described compounds, high-performance liquid chromatography (HPLC) was applied using a L-7100 pump from Hitachi (Japan) and a 785A UV−vis detector from Applied Biosystems (United States). Compounds were dissolved as 1 M stock solutions in 96% ethanol and diluted to 20 mM in LB. The solution was shaken vigorously for 2 h at 37 °C and then centrifuged for 15 min at 13000*g*. Afterward, a 20 μL aliquot was injected into a Phenomenex Luna C18 column (4.6 mm × 150 mm i.d.). The elution was performed at a flow of 0.8 mL/min using a mixture of water (A) and acetonitrile (B) with the following characteristics: 1 min isocratic flow with 50% B, from 1 to 15 min, B increased to 60%, 15−17 min, B increased to 100%, and finally, the column was washed with 100% B for 5 min. Detection was performed at 254 nm, and the area under the curve was compared to standard solutions of 1, 5, 10, and 20 mM in 96% ethanol to determine the amount dissolved in LB.

Growth Curves. Growth curves of bacteria in LB containing carvacrol and other carvacrol-related compounds were determined using a Bioscreen C OD reader (Oy Growth Curves AB Ltd., Helsinki, Finland) using software version 2.28. Stock preparations of 1 M of the tested compounds were made up in 96% ethanol and diluted in LB to the appropriate concentration. Final ethanol concentrations never exceeded 2%, to avoid an antibacterial effect of ethanol itself (observed at 4% and higher in our experimental conditions). After dilution, the solutions were shaken vigorously for 2 h at 37 °C to completely dissolve the compounds. In a typical experiment, aliquots of 100 μL of bacterial suspension containing 2×10^6 colony-forming units (CFU)/mL were added to 100 μL of LB containing the tested compound. For substances with low antimicrobial activity, the ratio of bacterial suspension and antimicrobial substance solution was changed to obtain a higher final concentration of active compounds, but the final density of bacteria was not altered. Bacterial densities used in the experiments were checked by plating out on LB agar plates for 24 h at 37 °C.

All wells were incubated for 18 h at 37 °C with continuous shaking. OD measurements (broad filter 420−580 nm) were obtained automatically at every 30 min. Each experiment was carried out in duplicate with 3−5 replicates each time. A positive growth control containing no EO components was run on every occasion, and negative controls containing no bacteria were included in each experiment. Fifty milliliters, and a 100-fold dilution thereof, of the wells where no visible growth had occurred were plated out for detection of viable bacteria. The minimal inhibitory concentration (MIC) value was defined as the lowest concentration where no increase in OD was observed after 18 h. The minimal bactericidal concentration (MBC) was defined as the lowest concentration where no viable bacteria were detected.

Cell Death Time Determination. Compounds dissolved in LB and bacterial suspensions were mixed as described above. After 1, 2, 5, 10, and 20 min, 100 μL was taken from the incubation mixture (after a short mix) and immediately diluted in cold minimal LB medium (1% LB). Subsequently, the samples were plated out on LB agar plates and incubated for 24 h at 37 °C prior to cell counting.

Effect of Compounds on the Membrane Potential. The effect of the compounds on the membrane potential was tested as described by Wu et al (*16*), with small modifications. Briefly, an overnight culture of *S. aureus* was harvested and washed twice in buffer A (5 mM HEPES, pH 7.2; 5 mM glucose). Subsequently, the bacterial suspension was diluted to 5×10^6 CFU/mL in buffer A. $DiSC_3$5 was added from a 2 M stock solution in 96% ethanol to a final concentration of 5 μM. The membrane potential was monitored with a Perkin-Elmer LS50B spectrofluorometer at 20 °C (excitation wavelength, 643 nm; emission wavelength, 666 nm). The bacteria were incubated for 1 h in the dark until a stable base signal was reached. Carvacrol and other compounds were added from a 1 M stock solution, and valinomycin (10 nM) was used as a positive control.

RESULTS

Solubility of Compounds. The solubility in LB at 37 °C of all tested compounds was determined using HPLC/UV and is shown in **Table 1**. As expected, the solubility correlated well with the hydrophilicity of the compounds (reflected in shorter retention times for the more hydrophilic compounds). The solubility of carvacrol using this method is slightly lower than that described for water, which could be due to the ingredients of LB. The solubility of *p*-cymene and 3,4-dimethylcumene was below the detection limit, which was determined at 0.05 mM. Therefore, the concentrations of 3,4-dimethylcumene and *p*-cymene described in the experiments below should not be read as true concentrations but more as the amount of 3,4-dimethylcumene or *p*-cymene present as an emulsion in the experiment. The solubility of 2-amino-*p*-cymene was com-

Table 1. Solubility of Carvacrol and Structurally Related Compounds in LB Medium[a]

compound	solubility in LB (mM)	retention time (min)
o-cresol	>20	5.2
3-isopropylphenol	14.4 ± 0.1	8.2
2-amino-*p*-cymene	6.4 ± 0.5	11.3
carvacrol	6.7 ± 0.4	11.5
p-cymene	ND[b]	21.3
3,4-dimethylcumene	ND[b]	21.9

[a] Solubility is shown as the average of triplicate measurements ± standard deviations. [b] Not detectable. The detection limit was 0.05 mM for both *p*-cymene and 3,4-dimethylcumene.

Table 2. MIC and MBC of Carvacrol and Related Compounds against *E. coli* and *S. aureus*

	mM			
	E. coli		*S. aureus*	
compound	MIC	MBC	MIC	MBC
carvacrol	1.2	1.3	1.7	1.8
3-isopropylphenol	1.7	1.8	2.6	3.0
o-cresol	3.0	3.4	3.0	3.6
2-amino-*p*-cymene	4.0	4.25	5.9	>6
p-cymene	>36	>36	>36	>36
3,4-dimethylcumene	>36	>36	>36	>36

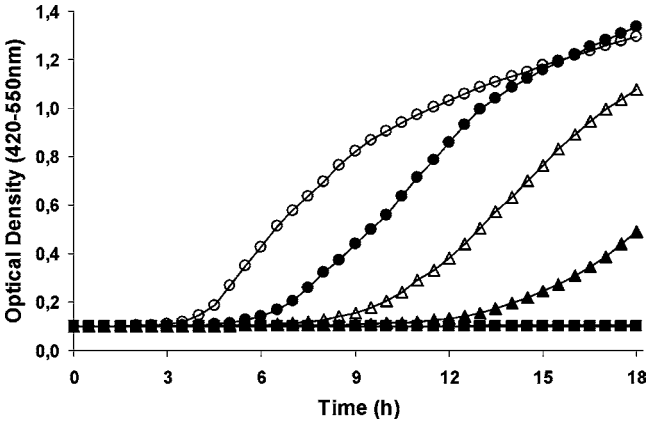

Figure 2. Representative growth curves of *S. aureus* in the presence of different concentrations of carvacrol. Key: 0 (○), 1.1 (●), 1.3 (△), 1.5 (▲), 1.7 (□), and 1.9 mM (■) carvacrol.

parable to carvacrol whereas 3-isopropylphenol and *o*-cresol had higher solubility in LB.

Growth Curves. The MIC and MBC were determined for all carvacrol-related compounds against *E. coli* and *S. aureus* using the dilution broth assay. An example of the MIC determination for carvacrol is given in **Figure 2**. It can be seen from this figure that increasing amounts of carvacrol (0−1.9 mM) led to extended lag phases and lower maximum growth rates. There was no growth detected after 18 h at 1.7 mM, and this was taken as the MIC. The MICs determined using this way for all compounds are shown in **Table 2**, which shows that carvacrol had the highest activity among the tested compounds. For all compounds except one (*o*-cresol), the MICs were approximately 1.5-fold lower against *E. coli* as compared to *S. aureus*. Removal of one of the side groups from carvacrol resulted in higher MIC values; however, the extent of decreased activity is remarkably different. Removal of the methyl group (3-isopropylphenol) or the isopropyl group (*o*-cresol) results in

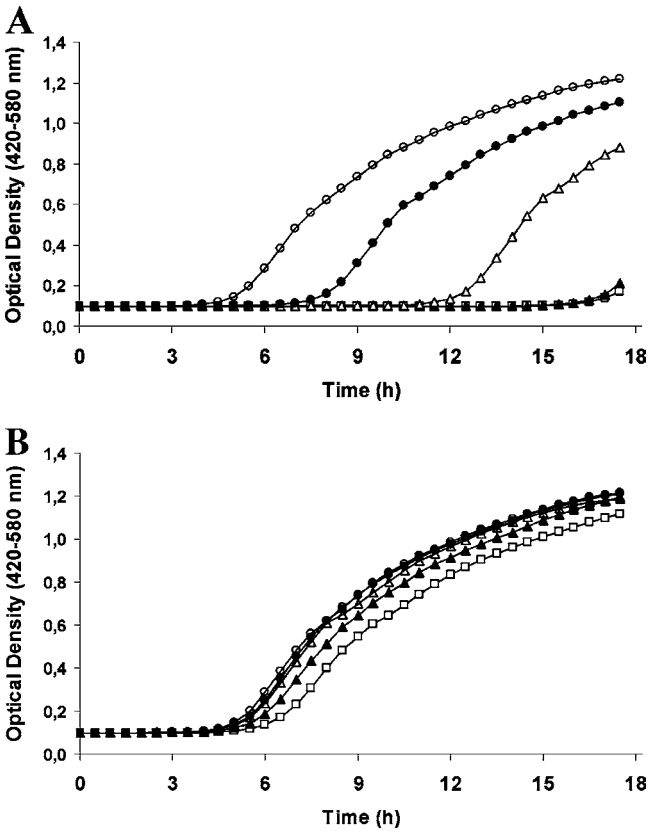

Figure 3. Representative growth curves of *S. aureus* in the presence of different concentrations of 3,4-dimethylcumene (**A**) and *p*-cymene (**B**). Key: 0 (○), 14 (●), 22 (△), 28 (▲), and 36 mM (□).

approximately a 2- and 2.5-fold increase, respectively. In contrast, removal of the hydroxyl substituent (*p*-cymene) resulted in a complete loss of any detectable activity. This loss of activity could be overcome by replacing the hydroxyl with an amino substituent (2-amino-*p*-cymene), whereas replacement with a methyl group (3,4-dimethylcumene) did not result in complete inhibition of growth at the concentrations tested.

The MBC values were slightly higher than the MIC values of all compounds; however, the MBC values of 2-amino-*p*-cymene varied between 6 and 14 mM for both *S. aureus* and *E. coli*. This high variation is probably due to the fact that the MBC is close to the maximum solubility of 2-amino-*p*-cymene in LB medium. Solubility problems therefore likely interfere with an accurate determination of the MBC. The average of the determined values would not represent an accurate MBC value and is therefore not shown in **Table 2**.

Despite the inability to detect a MIC, there was a small antimicrobial effect observed for 3,4-dimethylcumene against *S. aureus* but not against *E. coli*. As is depicted in **Figure 3A**, the lag phase at [3,4-dimethylcumene] > 14 mM is significantly increased, while an equal amount of ethanol or *p*-cymene (**Figure 3B**) at the same concentrations had no effect on the growth curve of *S. aureus*. The tested concentration of 3,4-dimethylcumene (and *p*-cymene) is higher than the solubility of these compounds in pure LB. However, the results show that increasing the amount of 3,4-dimethylcumene above the solubility limit increased the effect on the lag time of the growth curve. Apparently, even though all of the 3,4-dimethylcumene is in suspension instead of in solution, it can still interact with bacterial membranes at the interface of the "oily" 3,4-dimethylcumene drops. Addition of agar to a final concentration 0.1% (w/v) to increase viscosity and decrease phase separation of the

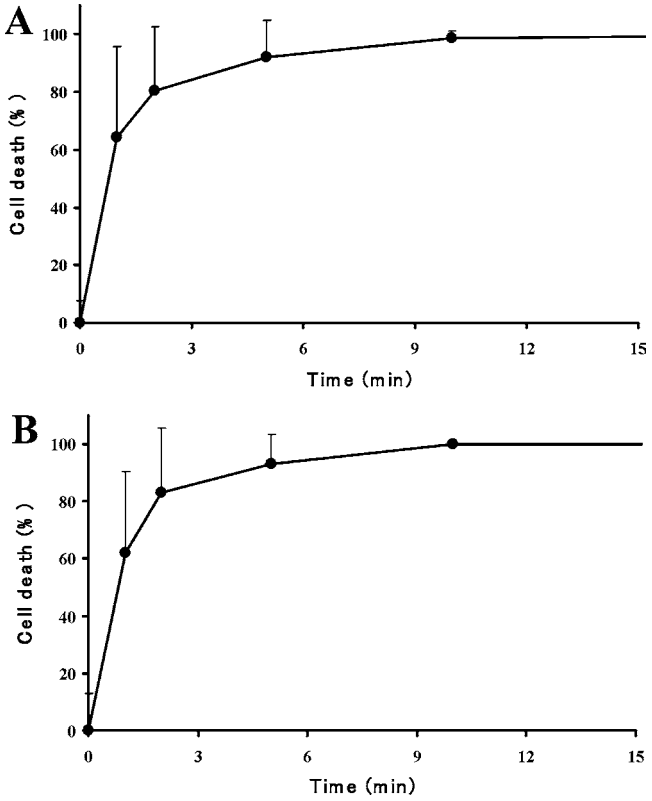

Figure 4. Effect of carvacrol (**A**) and 2-amino-*p*-cymene (**B**) on the number of viable *E. coli.* Means and standard deviations of triplicate experiments are shown.

mixture did not enhance the observed antimicrobial effects of either *p*-cymene or 3,4-dimethylcumene (results not shown).

Cell Death Time Determination. All structures containing antimicrobial activity showed similar fast killing kinetics. The kill curve for carvacrol and 2-amino-*p*-cymene against *E. coli* is shown in **Figure 4**. Within 10 min, >95% killing was achieved at 1.5× MIC concentrations. Similar kinetics were observed for 3-isopropylphenol and *o*-cresol (not shown). No killing was observed for 3,4-dimethylcumene even at the highest concentration tested, which indicates that the extended lag phase shown in **Figure 3** is not caused by partial killing of the bacteria. The killing kinetics were determined for several concentrations. As expected, higher concentrations of the active compounds resulted in increased killing of the microorganisms leading to 100% killing within 5 min for 3× MIC and 4× MIC concentrations (results not shown).

Disruption of Membrane Potential. Exposure of *S. aureus* to carvacrol led to a loss in membrane potential, as is shown in **Figure 5**. A concentration of 1× MIC was required to observe the increase in fluorescence resulting from the loss of membrane potential. At 0.5× MIC, a very low increase was observed. Values between 0.5 and 1.0× MIC were tested, but only at 1× MIC the extent of the fluorescence increase shown in the figure was observed. The other active compounds 2-amino-*p*-cymene and *o*-cresol had similar effects on the membrane potential: At 0.5× MIC, there was no increase or a small increase in fluorescence, while 1× MIC led to an increase of the fluorescence to a similar extent as carvacrol. Addition of extra carvacrol, valinomycin, or Triton X-100 did not lead to a further increase in fluorescence, indicating that the membrane potential could not be decreased further. Remarkably, a direct effect of all compounds on $DiSC_3 5$ in solution was detected, resulting in a decreased fluorescence if the compounds in solution could

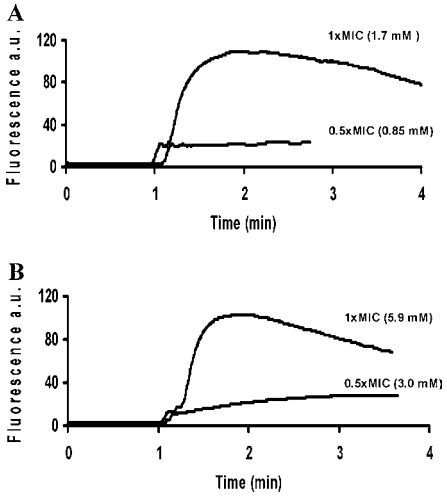

Figure 5. Effect of carvacrol (**A**) and 2-amino-*p*-cymene (**B**) on the membrane potential of *S. aureus.* Both compounds were added at 1 min. The membrane potential was monitored with the fluorescent probe $DiSC_3 5$. Fluorescence at 666 nm is depicted in arbitrary units.

interact with $DiSC_3 5$. This phenomenon can be seen in the curves shown in **Figure 5** where a decrease in fluorescence is observed after the initial rise due to the membrane disruption. This effect of decrease in fluorescence was also observed in a bacteria-free environment when carvacrol was directly added to $DiSC_3 5$ in solution (results not shown).

DISCUSSION

In this study, carvacrol and carvacrol-related compounds were tested with respect to their antimicrobial activities to define structural requirements for the antibacterial activity of carvacrol. The observed MIC value for carvacrol was comparable to other values previously reported against the same and other microorganisms (*14*, *17*−*23*). By removing the ring substituents of carvacrol, it was observed that all removals reduced the antimicrobial activity. The lowered activities of 3-isopropylphenol and *o*-cresol (removal of the aliphatic side chains of carvacrol) are probably due to the decrease in amphipatic features of these molecules, which may affect the initial interaction of these compounds with the bacterial membrane. This also explains the smaller effect of the methyl group as compared to the isopropyl group on the activity. No antimicrobial activity was observed for *p*-cymene using our experimental condition, as has been described by several researchers (*4*, *17*, *18*, *24*, *25*). However, some reports have shown weak activity of *p*-cymene (*4*, *26*, *27*). The lack of antimicrobial activity of *p*-cymene is usually attributed to the lack of a hydroxyl group (*4*, *15*). However, this explanation is too simple since the low solubility of this compound in aqueous solutions prevents a quantitative determination of its activity. The observation that *p*-cymene does not show activity in the common activity assays can therefore not be used to define a functional antimicrobial role of the hydroxyl group of carvacrol. The same argument holds true for the methyl ether of carvacrol, which has a similar hydrophobicity as compared with *p*-cymene.

Some researchers have indicated the problems of immiscibility of oils or EO compounds such as *p*-cymene with aqueous solutions and have used other methods to overcome them, such as the use of the pure compound in agar disk diffusion assays (*4*), the use of emulsions of the compound by vigorous shaking (*5*), or the addition of an emulsifier like Tween-20 (*28*) or agar (*17*) to the solution. Despite improvements in reproducibility,

these methods still do not suffice to quantify antimicrobial activity of the compounds. Zone inhibition in the agar-based technique will depend on the ability of the compound to diffuse through the agar medium, which will be dependent again on the hydrophobicity of the compound. (*24*, *28*). The addition of an emulsifier to the oil/water mixture can result in a more even distribution of compound over the aqueous solution. This can increase the activity of a compound because the contact area where compounds can interact with bacteria is increased. This was recently described for carvacrol emulsions (*17*), but no effect was seen in our current experiments for 3,4-dimethylcumene or *p*-cymene. Whether this is a compound specific feature is unknown. Despite the possible positive effect of emulsifiers on antimicrobial activity, an emulsion is on a molecular level not at all comparable to a true solution of a compound. In addition, the effect of the emulsifier itself on the antimicrobial activity should be accounted for. Therefore, in our opinion, no method has been described thus far that can quantitatively compare the antimicrobial activity of compounds with different hydrophobicity like *p*-cymene and carvacrol.

The use of 2-amino-*p*-cymene, an amino analogue of carvacrol, circumvents the described solubility problems and gives a more reliable indication of the necessity of the hydroxyl group of carvacrol for its antimicrobial activity. The hydrophobicity (reflected in the similar retention time in the HPLC experiments) and the spatial structure of these two compounds are highly similar. The observed MICs show that a hydrophilic group on the benzene ring is adequate for activity. The amphipathicity of compounds such as carvacrol and 2-amino-*p*-cymene could be a prerequisite to confer the optimal orientation in the membrane. The hydrophilic part of the molecule interacts with the polar part of the membrane, while the hydrophobic benzene ring and the aliphatic side chains are buried in the hydrophobic inner part of the bacterial membrane. However, even though the amino group restored most of the observed activity of carvacrol, the 3-fold increase in MIC for 2-amino-*p*-cymene indicates specific characteristics of the hydroxyl group of carvacrol. One possible explanation is the involvement of the hydroxyl group in the formation of hydrogen bonds, since amino groups have a significantly lower capacity to form these bonds. These hydrogen bonds could easily occur in the membrane with the phosphate or the ester linkages of phospholipids. This strong interaction could influence the interaction with and the orientation in the membrane. A second important aspect is the acidity of phenolic compounds as compared to an aniline-based compound such as 2-amino-*p*-cymene. Although only weakly acidic (pK_a slightly higher than 10 for alkylated phenols), the capacity to donate a proton could be involved in the working mechanism of carvacrol. The low activity of menthol (2-isopropyl-5-methylcyclohexanol) would confirm this involvement (*15*). Menthol is not acidic because of the lack of delocalized electrons in the ring structure that stabilize the resulting anion, which could explain the observed necessity of the delocalized electrons for antimicrobial activity (*4*, *15*). Ultee et al. proposed a mechanism of action for carvacrol based on this acidity (*15*). In this mechanism, the activity of carvacrol is related to the loss of the proton gradient, and thus the proton motive force, over the bacterial membrane. Briefly, carvacrol may diffuse back and forth through the bacterial membrane, while exchanging the acidic proton for another cation on the cytosolic side of the membrane and the opposite cation exchange at the exterior side. Although this mechanism could play a role in the antimicrobial action of carvacrol, the relatively high activity of 2-amino-*p*-cymene seems to rule out that this is the main mode of killing action for carvacrol. In addition, Sikkema et al. have shown that many hydrocarbons (non hydroxylic) with affinity for membranes have antimicrobial activity (*29*). Their general mode of action was related to membrane disruption leading to loss of the high impermeability of the membrane for protons and bigger ions. Although this does not rule out specific carvacrol-related antimicrobial activities, it does indicate that membrane disruption itself is a major factor in the antimicrobial effect of compounds such as carvacrol. The observed correlation, in this work, between MIC and membrane disruption favors this hypothesis. Besides the interaction with membrane phospholipids discussed above, an interaction of carvacrol with membrane proteins or intracellular proteineous targets has been suggested by some researchers (*4*, *30*, *31*). However, so far, no conclusive evidence has been presented. Given the numerous amount of possible targets and the ways the hydroxyl group could be involved, these options are not discussed here.

In summary, the activity of carvacrol was compared to structurally related (non-EO) compounds to detect the structural requirements for its antimicrobial activity. The activity of the tested compounds was related to the solubility in aqueous solution. The aliphatic side chains of carvacrol have a small effect on the activity of carvacrol probably by interacting with the bacterial membrane. The functional role of the hydroxyl group cannot be determined by simple deletion experiments because of solubility problems of *p*-cymene; therefore, the hydroxyl group was substituted for an amino group, which resulted in a 3-fold lower activity. Because this substitution does not affect the hydrophobicity, spatial structure, and solubility of the compound, it may be concluded that the hydroxyl group of carvacrol is not essential for activity but does have special features that add to the antimicrobial mode of action of carvacrol.

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