JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antibacterial Action of Anacardic Acids against Methicillin Resistant *Staphylococcus aureus* (MRSA)

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The structural and antibacterial activity relationship of 6-alk(en)ylsalicylic acids, also known as anacardic acids, was investigated against Gram-positive bacteria, emphasizing the methicillin resistant *Staphylococcus aureus* ATCC 33591 (MRSA) strain. The unsaturation in the alkyl side chain is not essential in eliciting activity but is associated with increasing the activity. The antibacterial activity of methicillin against MRSA strains was significantly enhanced in combination with $C_{12:0}$-anacardic acid, and the fractional inhibitory concentration index for this combination was calculated as 0.281. It appears that biophysical disruption of the membrane (surfactant property) is due to the primary response to their antibacterial activity, while biochemical mechanisms are little involved. The compounds possessing the similar log *P* values exhibit similar activity.

KEYWORDS: Antibacterial activity; methicillin resistant *Staphylococcus aureus* (MRSA); anacardic acids; surfactants; synergists; methicillin; fractional inhibitory concentration (FIC) index

INTRODUCTION

In our previous papers, the isolation of anacardic acids from two Anacardiaceae plants, *Anacardium occidentale* (*1*) and *Ozoroa mucronata* (*2*), and their diverse biological activities were reported. Anacardic acids isolated from these two plants are salicylic acid derivatives with a nonisoprenoid C_{15}-alkyl side chain that is monoenoic, dienoic, and trienoic at the C-8, -10, and -14 positions, all of cis configuration. Among the biological activities reported, the antimicrobial activity has been extensively investigated. The isolation of three antibacterial principles was achieved by bioassay-guided fractionation from the cashew nut shell liquid (CNSL) by recycle high-performance liquid chromatography (R-HPLC) using an ODS column. These antibacterial agents were identified by means of spectroscopic methods as anacardic acids, more specifically, 6-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]salicylic acid ($C_{15:3}$-anacardic acid) (**1**), 6-[8′(*Z*),-11′(*Z*)-pentadecadienyl]salicylic acid ($C_{15:2}$-anacardic acid) (**2**), and 6-[8′(*Z*)-pentadecenyl]salicylic acid ($C_{15:1}$-anacardic acid) (**3**), which were previously characterized from the CNSL (*3*, *4*). In addition, 6-pentadecylsalicylic acid ($C_{15:0}$-anacardic acid) (**4**) did not show any activity but was isolated from the same source in minute amounts by R-HPLC. This $C_{15:0}$-anacardic acid was also obtained by hydrogeneration of the mixture of anacardic acids (**1**−**4**) over Pd−C and tested for comparison.

Gellerman et al. (*5*) previously reported that a decrease in the number of double bonds in the side chain of the C_{15}-anacardic acids decreases the antibacterial activity. We also observed similar results against Gram-positive bacteria (*6*). However, the role of double bonds in eliciting activity was not precisely explained. The recent result that 6-dodecylsalicylic acid ($C_{12:0}$-anacardic acid) (**5**) showed equally potent activity as $C_{15:3}$-anacardic acid does not support the previous observation. The double bond in the side chain in **1**−**3** is not essential in eliciting antibacterial activity but is involved in increasing it to a large extent. On the other hand, the alk(en)yl chain length in anacardic acids seems to play an important role but the rational explanation remains obscure. In addition, $C_{15:3}$-anacardic acid showed bactericidal activity against *Staphylococcus aureus* ATCC 33591 (MRSA) strains and the bactericidal action of methicillin against these MRSA strains was dramatically enhanced through combination with this anacardic acid (*7*). The rationale for this combination remains unknown. Hence, further study of anacardic acids to gain new insights into their antibacterial action on a molecular basis was achieved.

MATERIALS AND METHODS

Chemicals. Natural C_{15}-anacardic acids (*1*) and their synthetic analogues (**1**−**8**) (*8*, *9*) were available from our previous works. 6-[8′-(*E*)-Pentadeceny]salicylic acid (**9**) and 6-[8′(*Z*)-heptadecenyl]salicylic acid (**10**) were provided by Prof. I. Green and Dr. T. Matsumoto, respectively. Salicylic acid and methicillin were purchased from Sigma Chemical Co. (St. Louis, MO). *N*,*N*-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ). Log *P* values were achieved by Chem Draw Pro version 4.5 (Cambridge Soft Co., Cambridge, MA) using Crippen's fragmentation (*10*).

Test Strains. The microorganisms, *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 12598, *S. aureus* ATCC 25923, *S. aureus* ATCC 33591 (methicillin resistant), *S. aureus* ATCC 33592 (gentamicin and methicillin resistant), *S. aureus* ATCC 11632 (penicillin resistant), *S. aureus* ATCC 29247, and *Micrococcus luteus* ATCC 4698 were purchased from American Type Culture Collection (Manassas,

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10.1021/jf034674f CCC: $25.00
Published on Web 11/13/2003

VA). *Pseudomonas aeruginosa* IFO 3080 was available from our previous work (*11*).

Medium. The NYG culture medium for the bacteria consisted of 0.8% nutrient broth (BD, Franklin Lake, NJ), 0.5% yeast extract (BD), and 0.1% glucose.

Antibacterial Assay. Broth macrodilution methods were used as previously described (*7*). Briefly, serial 2-fold dilutions of the test compounds were prepared in DMF, and 30 μL of each dilution was added to 3 mL of NYG broth. These were inoculated with 30 μL of a 2 day old culture of the test bacterium. After incubation of the cultures at 37 °C for 48 h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration of the test compound that demonstrated no visible growth. The minimum bactericidal concentration (MBC) was determined as follows. After the determination of the MIC, 100-fold dilutions with drug-free NYG broth from each tube showing no turbidity were incubated at 37 °C for 48 h. The MBC was the lowest concentration of the test compound that showed no visible growth in the drug-free cultivation. The concentration of DMF in each medium was always 1%, and the assays were performed at least in triplicate on separate occasions.

Combination Study. The combination data were obtained by the broth checkerboard method (*12*, *13*). A series of 2-fold dilutions of methicillin was tested in combination with concentrations of 2-fold dilutions of anacardic acids or their analogues. 6-*n*-Eicosylsalicylic acid ($C_{20:0}$) was not tested for the combination study because of its poor solubility in the culture media. The final concentration of DMF in each medium was 1%, which did not affect the growth of the test strain. In all cases, the highest concentration of each compound added to the bacterial culture was equal to the predetermined MIC. After the cultures were incubated at 37 °C for 2 days, the MICs were determined by using the method described above. The result of the checkerboard test was expressed as the fractional inhibitory concentration (FIC) index (*13*, *14*). In this method, synergism is defined as the FIC index of >0.5; additivity is defined as the FIC index of 0.5−1.0; and antagonism is defined as the FIC index of <1.0. The lowest FIC index from each checkerboard was recorded. The degree of synergism was compared also by the shape of the isobologram derived from a plot of the FICs produced by combinations of different concentrations of the two compounds (*12*, *14*). The inward bowing of the isobologram indicates synergism between two antimicrobial agents, and the isobologram bows more inward as the synergism is stronger.

RESULTS

The antibacterial activity of anacardic acids (**1**−**10**) (see **Figure 1** for structures) against *S. mutans* ATCC 25175 and MRSA was tested using a broth dilution method. The results are listed in **Table 1**. Among the compounds tested, $C_{15:3}$-anacardic acid (**1**), $C_{12:0}$-anacardic acid (**5**), and $C_{10:0}$-anacardic acid (**8**) were the most potent, each with an MBC of 6.25 μg/mL against MRSA. No differences in their MICs and MBCs were noted, suggesting that their activity is bactericidal. On the other hand, $C_{15:0}$-anacardic acid (**4**) did not exhibit any activity against MRSA up to 800 μg/mL. The result that $C_{12:0}$-anacardic acid and $C_{10:0}$-anacardic acid showed equally as potent activity as $C_{15:3}$-anacardic acid indicates that the double bond in the alkyl side chain is not essential. This conclusion does not support previous reports (*5*) but suggests that the alkyl chain length is significantly associated with the activity instead (*6*). In the case against *S. mutans*, $C_{15:2}$-anacardic acid (**2**) was the most effective with an MBC of 3.13 μg/mL, followed by $C_{15:3}$-anacardic acid and $C_{15:1}$-anacardic acid (**3**), each with an MBC of 6.25 μg/mL. Neither salicylic acid nor $C_{15:0}$-anacardic acid exhibited any activity up to 800 μg/mL. It appears that the compounds possessing the similar log *P* values exhibit similar MBC values (*15*), and 3.13 μg/mL against *S. mutans* seems to be the maximum bactericidal activity. Because of adaptability, *S. aureus* can easily develop resistance to commonly used antibiotics (*16*). There is a great need for effective antibacterial agents against *S. aureus* with new modes of action. Hence, further discussion is centered against the MRSA ATCC 33591 strain.

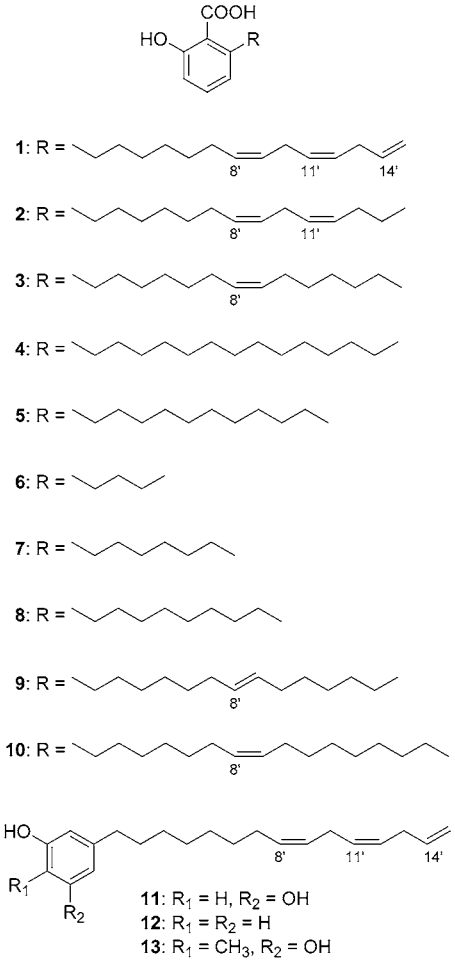

Figure 1. Chemical structures of anacardic acids and related compounds.

Table 1. Antibacterial Activity of Natural and Synthetic Anacardic Acids and Salicylic Acid

compds tested	MIC and MBC (μg/mL)[a] *S. mutans*[b]	*S. aureus*[c]	log *P*
	natural		
1 ($C_{15:3}$)	1.56 (*6.25*)	6.25 (*6.25*)	6.62
2 ($C_{15:2}$)	3.13 (*3.13*)	12.5 (–)	6.89
3 ($C_{15:1}$)	6.25 (*6.25*)	100 (–)	7.21
4 ($C_{15:0}$)	>800 (–)	>800 (–)	7.53
10 ($C_{17:1}$)	>800 (–)	>800 (–)	8.05
	synthetic		
6 ($C_{5:0}$)	200 (–)	100 (–)	3.36
7 ($C_{8:0}$)	50 (–)	12.5 (–)	4.61
8 ($C_{10:0}$)	3.13 (*12.5*)	6.25 (*6.25*)	5.44
5 ($C_{12:0}$)	1.56 (*6.25*)	6.25 (*6.25*)	6.28
9 ($C_{15:1}$)[d]	6.25 (*12.5*)	100 (–)	7.21
salicylic acid	>800 (–)	400 (–)	2.25

[a] Numbers in italic type in parentheses are MBCs; –, not tested. [b] ATCC 25175. [c] ATCC 33591 (MRSA). [d] *E*-isomer.

The stereochemistry of the double bonds in the alkyl side chain in C_{15}-anacardic acids (**1**−**3**) needs to be checked if they are associated with the activity. In the case of $C_{15:0}$- and $C_{12:0}$-anacardic acids, their saturated alkyl side chains exist usually in the extended form, which requires the least amount of energy to maintain. On the other hand, the unsaturated alkyl side chains in C_{15}-anacardic acids (**1**−**3**) have a bend of about 30° in the hydrocarbon side chain, imposed on the molecule by the cis

Table 2. Antibacterial Activity of $C_{15:3}$-Anacardic Acid (**1**), $C_{12:0}$-Anacardic Acid (**5**), and Salicylic Acid against the Six Selected Strains of *S. aureus*

strains tested	MIC and MBC (μg/mL)[a]				salicylic acid
	penicillin G	methicillin	**1**	**5**	
ATCC					
12598[b]	0.049 (*6.25*)	1.56 (>*6.25*)	6.25 (*6.25*)	6.25 (*6.25*)	>800 (–)
25923[b]	0.049 (*3.13*)	1.56 (–)	3.13 (*6.25*)	1.56 (*3.13*)	>800 (–)
33591[c]	>800 (–)	800 (>*800*)	6.25 (*6.25*)	6.25 (*6.25*)	>800 (–)
33592[c]	>800 (–)	800 (>*800*)	6.25 (*12.5*)	6.25 (*6.25*)	>800 (–)
11632[d]	800 (–)	1.56 (–)	3.13 (–)	3.13 (–)	>800 (–)
28247[d]	>800 (–)	3.13 (*50*)	12.5 (–)	6.25 (–)	>800 (–)

[a] Numbers in italic type in parentheses are MBCs; –, not tested. [b] Methicillin susceptible strain. [c] Methicillin resistant strain. [d] Penicillin resistant strain.

configuration of the double bond. Two double bonds in the cis configuration in $C_{15:3}$- and $C_{15:2}$-anacardic acids (**1**, **2**) create more bends and significantly shorten the side chain length. The magnitude of the disordering effect is a function of the length of the chain distal to the double bond. For example, $C_{15:0}$-anacardic acid lacks the double bond in the side chain, which has been shown to be critical for imparting the antibacterial activity. Therefore, it seems that the presence of the 8,9-cis double bond is a specific structural requirement. Thus, the cis double bond at C-8 position in the C_{15}-alkyl side chain significantly affects their molecular shape. However, the observation is that 6-[8′(*E*)-pentadeceny]salicylic acid ($C_{15:1}$) (**9**) exhibited similar activity against the two bacteria. The conversion of the 8,9-cis double bond to the 8,9-trans did not eliminate activity, indicating that the cis double bond in the side chain is not essential to elicit activity. Moreover, 6-[8′(*Z*)-heptadecenyl]salicylic acid ($C_{17:1}$) (**10**) did not exhibit any activity up to 800 μg/mL. It is evident that the molecular shape does not appear to be a major contributor to the activity. Overall, the double bond in the alkyl side chain is not essential but is involved with increasing activity.

The antibacterial activity of $C_{15:3}$-anacardic acid (**1**) and $C_{12:0}$-anacardic acid (**5**), penicillin G, and methicillin were tested against the six selected *S. aureus* strains for comparison. The results are listed in **Table 2**. Both $C_{15:3}$- and $C_{12:0}$-anacardic acids were effective against all of the strains of *S. aureus* tested, with the MICs ranging from 1.56 to 12.5 μg/mL. The activity of $C_{12:0}$-anacardic acid is slightly more potent than that of $C_{15:3}$-anacardic acid against some strains tested. Two strains of methicillin resistant *S. aureus* were also resistant to penicillin G. Overall, the MICs of $C_{15:3}$- and $C_{12:0}$-anacardic acids did not considerably differ among the strains tested.

The antibacterial activity of methicillin against MRSA strains was previously reported to be enhanced in combination with $C_{15:3}$-anacardic acid (*7*). For example, the MIC of methicillin was lowered from 800 to 25 or 3.13 μg/mL against MRSA ATCC 33591 in combination with 1.56 (equivalent to 1/4MIC) or 3.13 μg/mL (1/2MIC) of $C_{15:3}$-anacardic acid, respectively. The FIC index for this combination was calculated as 0.281 (*13*, *14*). In addition, the time-kill curve experiment confirmed that the combinations were also bactericidal. As illustrated in **Figure 2**, the equally potent synergistic effect on antibacterial action was observed in combination with $C_{12:0}$-anacardic acid, indicating that the double bond is not essential in eliciting the synergistic activity. The FIC index for this combination was also calculated as 0.281. The synergism with methicillin was affected by the alkyl chain length of anacardic acids. The FIC index decreased with increasing the alkyl chain length. In connection with this, the FIC index of $C_{5:0}$-anacardic acid (**6**) was calculated as 0.563, indicating that the combination was only additive. It seems that a more lipophilic anacardic acid showed a more potent synergistic effect. The rationale for this effect remains obscure.

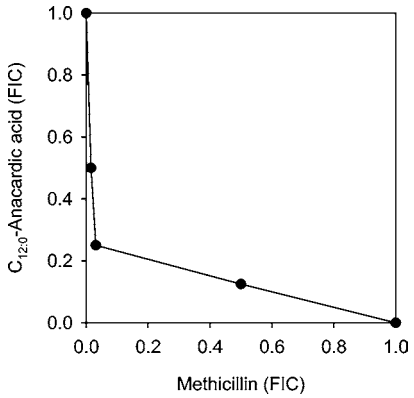

Figure 2. Resulting isobologram of the MICs obtained with combinations of methicillin and $C_{12:0}$-anacardic acid against MRSA ATCC 33591. The FIC index was calculated with the MICs of the combined compounds that exhibited the best antibacterial combination effect. Data were obtained by the checkerboard broth dilution technique at 37 °C. Each plot is the mean of triplicate determinations.

Table 3. NADH Oxidase[a] Inhibitory Activity of Anacardic Acids and Related Compounds

compds tested	IC_{50} (μg/mL)
anacardic acid	
1 ($C_{15:3}$)	1.3
2 ($C_{15:2}$)	5.4
3 ($C_{15:1}$)	2.1
4 ($C_{15:0}$)	1.5
5 ($C_{12:0}$)	>30
salicylic acid	>30
cardol (**11**)	8.2
cardanol (**12**)	>30
methylcardol (**13**)	>30

[a] Prepared from *M. luteus* ATCC 4698 cell membrane.

C_{15}-Anacardic acids (**1**−**4**) and their selected analogues were tested for their effects on the bacterial respiratory system. $C_{15:3}$-anacardic acid (**1**) inhibited oxygen consumption of *M. luteus* ATCC 4698 and *P. aeruginosa* IFO 3080 cells when the suspensions prepared from these bacterial cells were incubated with $C_{15:3}$-anacardic acid. In addition, anacardic acids inhibited *M. luteus* and *P. aeruginosa* NADH oxidase by a membrane fraction prepared from the same bacterial cells. The assay was carried out as previously described (*11*), and the results observed indicate that anacardic acids inhibit the bacterial membrane respiratory chain as listed in **Table 3**. It should be noted that *M. luteus* ATCC 4698 and *P. aeruginosa* IFO 3080 strains used for the experiment are aerobic bacterium. The respiratory inhibition causes the bacterial cell death due to the lack of anaerobic fermentative ability. At a glance, the antibacterial activity of anacardic acids comes, at least in part, from their ability to inhibit respiratory chain enzyme activity. All C_{15}-anacardic acids (**1**−**4**) inhibit the oxidation of NADH by a membrane fraction prepared from *M. luteus* cells, indicating that anacardic acids inhibit respiratory chain enzyme activity. Interestingly, salicylic acid did not exhibit respiratory inhibition, indicating that the C_{15}-alkyl side chain is important to elicit this inhibitory activity. However, $C_{12:0}$-anacardic acid did not inhibit the bacterial NADH oxidase up to 30 μg/mL, while $C_{15:3}$-cardol (**11**) inhibited this NADH oxidase. In addition, neither $C_{15:3}$-

cardanol (**12**) nor $C_{15:3}$-methylcardol (**13**) exhibited the NADH oxidase inhibition activity up to 30 μg/mL. The results obtained are consistent with the previous reports that anacardic acids exhibit uncoupling effects on oxidative phosphorylation of rat liver mitochondria using succinate as a substrate (*17*, *18*). Apparently, the number of double bonds in the side chain is not directly associated with the respiratory inhibition activity, but the alkyl side chain length appears to be related to the activity. It is worth noting that C_{15}-anacardic acids also inhibited NADH oxidase in bovine liver mitochondria, although to a lesser extent.

DISCUSSION

The bactericidal effect of $C_{15:3}$-anacardic acid against two MRSA strains, ATCC 33591 and ATCC 11632, was previously confirmed by the time-kill curve experiment. In both cases, lethality occurs quickly, within the first 2 h after the addition of $C_{15:3}$-anacardic acid. In addition, $C_{15:3}$-anacardic acid was found to exhibit bactericidal activity against both MRSA strains at any growth stage and also even when cell division was inhibited by chloramphenicol. It is thus not likely that the reduced bacterial viability is due to interaction with synthesis of macromolecules such as DNA, RNA, and proteins. These results most likely indicate that the bactericidal activity of $C_{15:3}$-anacardic acid against *S. aureus* is associated with the disruption of the membrane (*7*).

It appears that anacardic acids primarily act as a surfactant (physical disruption of the membrane), but biochemical mechanisms are barely involved in eliciting activity. The following needs to be taken into consideration. First, C_{15}-anacardic acids (**1**−**4**) have been found to inhibit bacterial respiration and inhibition of the respiratory chain, which is known to result in cell toxicities. The respiratory inhibition mechanism is not likely to be the primary mode of action of anacardic acids because all C_{15}-anacardic acids exhibited similar respiratory inhibition but $C_{15:0}$-anacardic acid did not show any antibacterial activity. However, the process by which anacardic acids reach to the action sites in living organisms is usually neglected in the cell-free experiment. On the basis of the data obtained, it can be assumed that the amphipathic anacardic acids are able to enter into the membrane lipid bilayers where various enzymes, especially components of energy converting systems such as electron transport chains (ETCs) and ATPases, are embedded. It should be kept in mind, however, that the ETC and ATPases involved in the respiratory chain are located at the inner face of the cytoplasmic membrane. The amphipathic anacardic acids entered into the lipid bilayers may disrupt the ETC and/or ATPases as surfactants. Hence, the possibility that anacardic acids first disrupt the membrane and then inhibit the respiratory chain cannot be entirely ruled out. Second, $C_{15:0}$-anacardic acid has previously been reported to show high selectivity toward Fe^{2+} and Cu^{2+} (*19*). This implicates that metal ions may have a role in antimicrobial activity by reducing their availability for bacteria (*20*). However, this possibility is unlikely since $C_{15:0}$-anacardic acid did not show any antibacterial activity up to 800 μg/mL. Furthermore, both $C_{15:3}$-cardol (**11**) and $C_{15:3}$-cardanol (**12**), isolated from the same cashew nut shell oil, showed similar antibacterial activity (*6*, *21*), indicating that the chelation is not essential to elicit activity. Third, anacardic acids have previously been reported to inhibit various enzymes. The β-lactamase inhibitory activity is remarkable, although they were not directly tested against this specific enzyme isolated from MRSA (*22*). This may explain how $C_{15:3}$-anacardic acid enhances bactericidal activity of methicillin against MRSA strains. In addition, anacardic acids were reported to inhibit lipid synthesis of bacterial cells by inhibiting glycerol-3-phosphate dehydrogenase (*23*). The relevance of the in vitro experiments in simplified systems to the complex interaction between anacardic acids and bacterial system needs to be carefully considered.

The current study indicates that anacardic acids seem to target the extracytoplasmic region and thus do not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. There still seems to be a lack of important knowledge for the design of effective antibacterial agents. The unsaturation in the alkyl side chain is not essential in eliciting activity but is associated with increasing it. A similar phenomenon among C_{18}-fatty acids is well-known (for example, linoleic acid vs stealic acid) (*24*, *25*), but the precise explanation for this still remains unknown. The lipophilicity of molecules is known to affect biological activities to a large extent (*26*). For instance, the increased lipophilicity of molecules decreases their solubility in water-based test media. In other words, the hydrophobic portion of molecules plays an important role in eliciting activity but the rationale for this is still poorly understood. In brief, $C_{15:3}$- and $C_{12:0}$-anacardic acids appear to possess the optimum balance of the head and tail structure. The observation that a decrease in the number of double bonds in the side chain of the C_{15}-anacardic acids decreases the antibacterial activity may be explained by the knowledge that the introduction of unsaturation or branching into the hydrophobic group is known to increase the solubility of the surfactant in water (*27*). As long as anacardic acids are compared, it may be logical to assume that a compound with a large alk(en)yl group and log *P* value around 6.5 should exhibit the most potent antibacterial activity against *S. aureus*.

Last, C_{15}-anacardic acids characterized from the cashew apple (*28*) were previously described to inhibit the growth of *Helicobacter pylori* (*29*), which is now considered to cause acute gastritis. *S. aureus* is one of the main bacteria that causes food poisoning, and *S. mutans* is the bacterium responsible for dental caries. Antibacterial agents from a regularly consumed fruit may be superior as compared to nonnatural products.

ACKNOWLEDGMENT

We are grateful to Dr. H. Muroi, Dr. M. Himejima, Dr. S. H. Lee, and Dr. A. Kubo for performing the antimicrobial assay at an earlier stage of the work and Dr. H. Haraguchi for performing the respiratory inhibition assay.

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Received for review June 24, 2003. Revised manuscript received October 9, 2003. Accepted October 10, 2003.

JF034674F