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Synergistic effects of anacardic acids and methicillin against methicillin resistant *Staphylococcus aureus*

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Abstract—The synergistic effects of 6-alk(en)ylsalcylic acids, also known as anacardic acids, in combination with methicillin against *Staphylococcus aureus* ATCC 33591 (MRSA) was investigated. The double bond in C_{15}-anacardic acids is not essential in eliciting the antibacterial activity but is associated with increasing the activity. The synergistic effects decreased with increasing the number of double bonds in the alkyl chain. On the other hand, the antibacterial activity of anacardic acids possessing different alkyl chain lengths against the same MRSA strain was found to be a parabolic function of their lipophilicity and maximized with the alkyl chain length of C_{10} and C_{12}. Notably, the synergistic effects were noted to increase with increasing the alkyl chain length.

1. Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) represents a therapeutic problem of increasing importance, especially in hospital patients.[1–4] In our continuing search for anti-MRSA agents from botanical sources, 6-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]salicylic acid, ($C_{15:3}$-anacardic acid) (**1**) (see Fig. 1 for structure), was reported to be an excellent anti-MRSA agent.[5] $C_{15:3}$-Anacardic acid rapidly killed MRSA and no viable cells were detected after 6 h of exposure to 6.25 μg/mL of this 6-alkenylsalicylic acid. In addition, a synergistic effect was observed in the combination of $C_{15:3}$-anacardic acid and methicillin, and the minimal inhibitory concentration (MIC) of methicillin was decreased from 800 to 1.56 μg/mL for MRSA ATCC 33591 by combining with 3.13 μg/mL (equivalent to 1/2MIC) of $C_{15:3}$-anacardic acid. Anacardic acids are salicylic acid derivatives with a non-isoprenoid alk(en)yl side chain and the influence of the length or unsaturation of the side chain on the synergistic effects is unknown. This prompted us to study the structural criteria to gain new insights into the synergistic effect on a molecular basis. The synergistic effects of the four natural C_{15}-anacardic acids (**1**–**4**) and a series of their synthetic analogues (**5**–**10**) in combination with methicillin against MRSA ATCC 33591 were evaluated by the broth checkerboard method.[6,7]

2. Results

The antibacterial activity of C_{15}-anacardic acids (**1**–**4**) isolated from the cashew *Anacardium occidentale* (Anacardiaceae) apple against MRSA ATCC 33591 was tested prior to the combination experiment. The results are listed in Table 1. Among these four C_{15}-anacardic acids, $C_{15:3}$-anacardic acid (**1**) exhibited the most potent activity against this MRSA strain with an MIC of 6.25 μg/mL whereas $C_{15:0}$-anacardic acid (**4**) did not show any activity up to 1600 μg/mL. It is evident that the activity increases with increasing the number of double bonds in their alkyl chain. Although the rationale for this remains unclear, it may be explained at least in part 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 and, as a result, increase the activity.[8]

Subsequently, the antibacterial activity of their synthetic analogues (**5**–**10**) differing in the alkyl chain length was also tested against the same MRSA strain for comparison. The results are listed in Table 2. The activity was significantly affected by the lengths of the alkyl chain. It appears that the activity obtained was a parabolic function of their lipophilicity and maximized with alkyl

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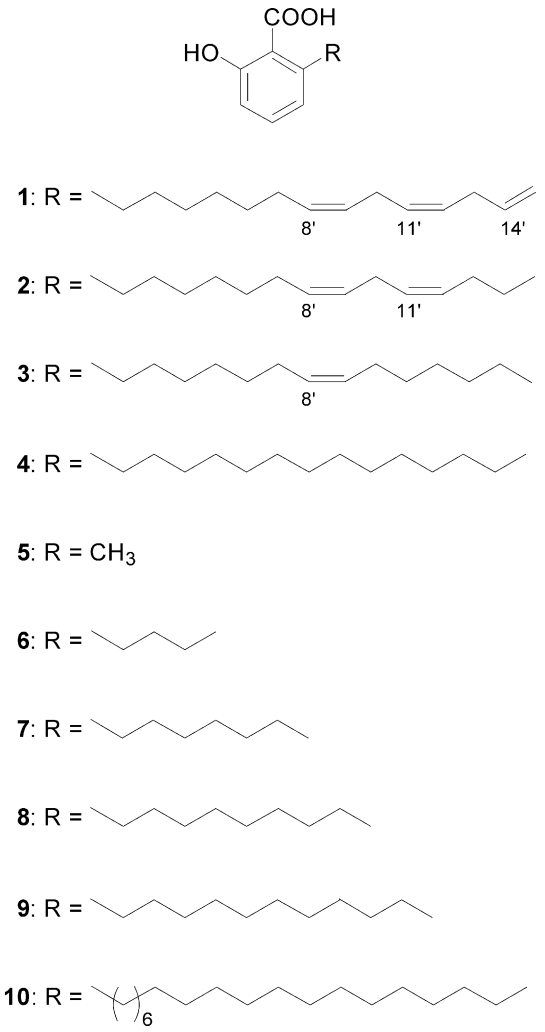

Figure 1. Structures of natural (**1**–**4**) and synthetic (**5**–**10**) anacardic acids.

Table 1. Antibacterial activity of anacardic acids (**1**–**4**) alone and in combination with methicillin against methicillin resistant *S. aureus* (MRSA) ATCC 33591

Anacardic acids	MIC (μg/mL)			
	Alone	Combination[a]		FIC index
		Anacardic acid	Methicillin[b]	
1: $C_{15:3}$	6.25	1.56	25	0.281 (S)[c]
2: $C_{15:2}$	12.5	1.56	50	0.187 (S)
3: $C_{15:1}$	100	3.13	25	0.063 (S)
4: $C_{15:0}$	1600	50	25	0.063 (S)

[a] This MIC by combination study expresses the concentration at FIC index obtained.
[b] The MIC of methicillin alone was 800 μg/mL.
[c] S, the combination was interpreted as synergistic. See Experimental.

chain lengths of C_{10} and C_{12}. The antibacterial activity of anacardic acids (**5**–**10**) was greater as the length of the alkyl chain was longer, and the maximum activity was observed in $C_{10:0}$-anacardic acid (**8**) and $C_{12:0}$-anacardic acid (**9**), each with an MIC of 6.25 μg/mL. The activity drastically dropped above these alkyl chain lengths, and $C_{15:0}$-anacardic acid (**4**) and $C_{20:0}$-anacardic acid (**10**) had no activity up to 1600 μg/mL. In brief, the results obtained indicates that the double bond in the alkyl side chain is not essential in eliciting the activity but is involved with increasing the activity.

The combination effects of natural C_{15}-anacardic acids (**1**–**4**) and methicillin were tested against MRSA ATCC 33591 by the checkerboard method. The results are listed in Table 1. The fractional inhibitory concentration (FIC) indices for all combinations were below 0.5, indicating synergistic interactions. There was a clear correlation between the FIC indices and the number of double bonds in the alkyl chain of anacardic acids. As far as C_{15}-anacardic acids are compared, the greatest synergism was observed in the combination of methicillin and $C_{15:0}$-anacardic acid (**4**), and the least synergism was observed in the combination with $C_{15:3}$-anacardic acid (**1**). While the MIC of $C_{15:1}$-anacardic acid was 100 μg/mL and that of methicillin was 800 μg/mL against MRSA ATCC 33591, the equal effect was achieved by 3.13 μg/mL of $C_{15:1}$-anacardic acid in combination with 25 μg/mL of methicillin. In other words, the MIC of methicillin was lowered from 800 to 25 μg/mL in combination with 3.13 μg/mL (equivalent to 1/32MIC) of $C_{15:1}$-anacardic acid. The activity of methicillin was enhanced 32-fold by combining with $C_{15:1}$-anacardic acid, and vice versa. Furthermore, 50 μg/mL of $C_{15:0}$-anacardic acid in combination with 25 μg/mL of methicillin inhibited the growth of this bacterium, although $C_{15:0}$-anacardic acid alone did not exhibit any activity up to 1600 μg/mL. Isobolograms constructed for the four natural C_{15}-anacardic acids in combinations with methicillin were shown in Figure 2. The synergistic effects of C_{15}-anacardic acids increased with decreasing the number of double bonds in the alkyl chain, and the synergism was the greatest in the combination of $C_{15:0}$-anacardic acid and methicillin among all tested combinations.

The synergism with methicillin was also affected by the alkyl chain length of anacardic acid analogues (**5**–**10**). Notably, the FIC index decreased with increasing the alkyl chain length as listed in Table 2. Thus, the greatest synergism was observed in the combination of $C_{12:0}$-anacardic acid and methicillin. The isobolograms indicate a clear relationship between the alkyl chain length of anacardic acid analogues and their combination effects with methicillin. The isobologram bows more inward as the length of the alkyl chain of these compounds becomes longer as shown in Figure 3. On the basis of the result obtained, an interesting question if a more lipophilic $C_{20:0}$-anacardic acid exhibits more potent synergistic effects needs to be answered. However, it could not be tested because of its poor solubility in the water based test medium.

3. Discussion

The antibacterial activity of anacardic acids and their synergistic effects in combination with methicillin against MRSA strains are related to both the length and

Table 2. Antibacterial activity of salicylic acid and anacardic acids (**5–10**) alone and in combination with methicillin against methicillin resistant *S. aureus* (MRSA) ATCC 33591

Compds Tested	MIC (μg/mL)			
	Alone	Combination[a]		FIC index
		Anacardic acids	Methicillin[b]	
Salicylic acid	400	200	200	0.750 (A)[c]
5: C_1	400	200	100	0.625 (A)
6: $C_{5:0}$	100	50	50	0.563 (A)
7: $C_{8:0}$	12.5	3.13	200	0.500 (S)
8: $C_{10:0}$	6.25	1.56	200	0.500 (S)
9: $C_{12:0}$	6.25	1.56	25	0.281 (S)
10: $C_{20:0}$	1600	—[d]	—	—

[a] This MIC by combination study expresses the concentration at FIC index obtained.
[b] The MIC of methicillin alone was 800 μg/mL.
[c] The combination was interpreted as additive (A) or synergistic (S). See Experimental.
[d] Not tested.

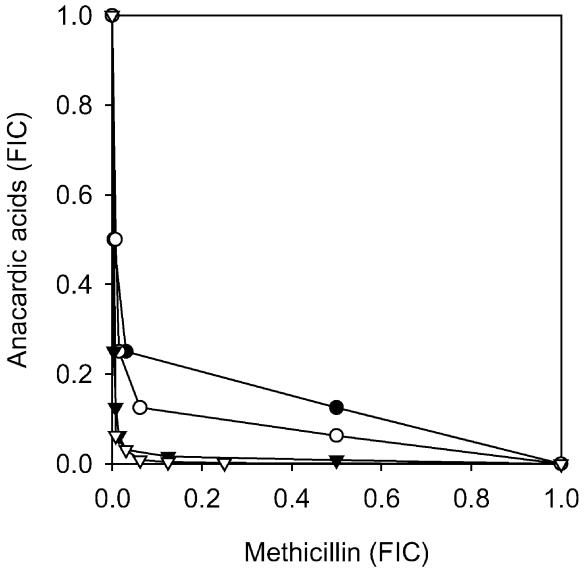

Figure 2. Isobolograms of the associations of C_{15}-anacardic acids (**1–4**) and methicillin against methicillin resistant *S. aureus* ATCC 33591. Filled circle, $C_{15:3}$-; circle, $C_{15:2}$-; filled triangle down, $C_{15:1}$-; triangle down, $C_{15:0}$-anacardic acid.

unsaturation of the alkyl side chain. The antibacterial activity of anacardic acids against MRSA increased with increasing the alkyl chain length up to a maximum at around 10–12 carbon atoms, and then dropped with the addition of further carbons to the chain. Similar relationships between the activity of antimicrobial compounds and their lipophilicity were previously observed with some membrane active antimicrobial agents, such as alcohols and quaternary ammonium compounds.[9–13] Daoud et al.[12] reported that the antimicrobial activity of a series of alkyldimethylbenzylammonium chlorides was a parabolic function of their lipophilicity and maximized with alkyl chain lengths between C_{12} and C_{16}. The penetration of these

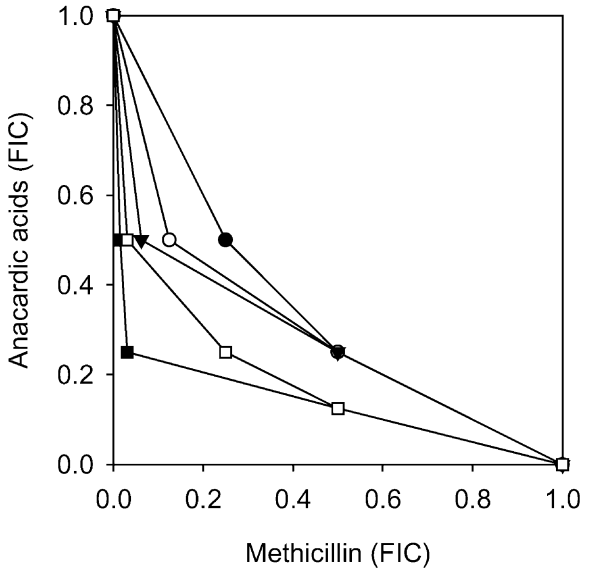

Figure 3. Isobolograms of the associations of anacardic acids (**5–10**) and methicillin against methicillin resistant *Staphylococcus aureus* ATCC 33591. Filled rectangle, $C_{12:0}$-; rectangle, $C_{10:0}$- and $C_{8:0}$- ; filled triangle down, C_5-; circle, C_1-anacardic acids and filled circle, salicylic acid.

compounds through cell membranes depends on their lipophilic properties. Substances with low oil solubility would be unable to cross the lipophilic barriers and remain localized in the first aqueous phase they contact. Conversely, those with high oil solubility would remain localized in the lipid regions.[14] Somewhere between these extremes there would be an optimum point of lipophilicity for traversing the cells barriers. This explanation could be applied in the case of anacardic acids. Anacardic acid with alkyl chain length between C_{10} and C_{12} appears to possess the optimum balance between hydrophilicity and lipophilicity to penetrate the cell membranes of MRSA. In addition to the length, the volume of lipophilic portions, which is altered by the position, number, and stereochemistry of double bonds, also affects the activity.[15,16] Since these structural parameters for the activity is important to selecting the proper anti-MRSA agents, more systematic studies are needed.

The structure–antibacterial activity relationship (SAR) of anacardic acids in combination with methicillin were somewhat different from those for a single compound against MRSA. Synergistic effects of anacardic acids in combination with methicillin increased with decreasing unsaturation of the alkyl side chain, and with increasing the alkyl chain length up to 15 carbon atoms. However, the mechanisms of the synergism observed between anacardic acids and methicillin is still unclear. The previous report that anacardic acids inhibit β-lactamase is remarkable, although they were not directly tested against this specific enzyme isolated from MRSA.[17] This may explain how anacardic acid ($C_{15:3}$) enhances bactericidal activity of methicillin against MRSA strains but needs to be confirmed.[18]

Overall, the synergistic effects of C_{15}-anacardic acid decreased with increasing the number of double bonds

in the alkyl chain ($C_{15:0} > C_{15:1} > C_{15:2} > C_{15:3}$), although their antibacterial activity increased with increasing the number of the double bonds in the alkyl chain ($C_{15:3} > C_{15:2} > C_{15:1} > C_{15:0}$). This does not follow the previous suggestion that the antibacterial agents used for combination study need to be bactericidal,[19] because $C_{15:0}$-anacardic acid did not show any antibacterial activity up to 1600 μg/mL. The precise explanation for this remains unknown. 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.[8]

Combinations of two or more compounds are generally superior to the use of a single compound, especially for the treatment of serious infections caused by antibiotics-resistant bacteria. The ability of anacardic acids to lower the MIC of methicillin for MRSA is useful property and these combinations could decrease the emergence of MRSA. Studies on the anti-MRSA mechanisms and in vivo efficacy of anacardic acids, alone and in combination with methicillin, deserve further investigation.

4. Experimental

4.1. Chemicals

C_{15}-Anacardic acids (**1**–**4**) were available from our previous works.[20] Since $C_{15:0}$-anacardic acid **4** was isolated only in min amounts, it was also derived by hydrogenation of the mixture of anacardic acids (**1**–**4**) over Pd-C. The serial analogues of anacardic acid, 6-alkylsalicylic acids (**5**–**10**), were previously synthesized.[21] Salicylic acid and methicillin were obtained from Sigma Chemical Co. (St. Louis, MO). For the antibacterial experiments, all compounds, except methicillin, were first dissolved in *N,N*-dimethylformamide (DMF) (EM Science, Gibbstown, NJ), and methicillin was dissolved in distilled water. The concentration of DMF in the medium was always 1%.

4.2. Test organism and medium

Methicillin resistant *S. aureus* ATCC 33591 was obtained from American Type Culture Collection (Manassas, VA). Antibacterial assay was performed using NYG broth, consisting of 0.8% nutrient broth (BD, Fkanklin Lake, NJ), 0.5% yeast extract (BD), and 0.1% glucose.

4.3. MIC determination

The MICs were determined by the broth dilution method as previously described.[5,22] The MIC was defined as the lowest concentration of a test compound resulting in complete inhibition of visible growth after 2 days of incubation at 37 °C. Because of solubility limitation of the samples in DMF and/or the water based medium, the highest concentration tested in the assay was 1600 μg/mL. The MIC of each compound was determined at least twice.

4.4. Combination studies

The combination data were obtained by the broth checkerboard.[6,7] A series of twofold dilutions of methicillin was tested in combination with concentrations of twofold 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 medium. The final concentration of DMF in each medium was 1%, which did not affect growth of the test strain. In all cases, the highest concentration of each compound added to the bacterial culture was equal to the pre-determined MIC. After incubation at 37 °C for 2 days, the MICs were determined by using the method described above. The result of checkerboard test was expressed as the FIC index.[7,23] In this method, synergism is defined as an FIC index of <0.5; additivity as an FIC index of 0.5-1.0; and antagonism as an 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.[6,23] The isobologram bowing inward indicates synergism between two antimicrobial agents, and the isobologram bows more inward as the synergism is stronger.

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