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Molecular design of anti-MRSA agents based on the anacardic acid scaffold

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Abstract—A series of anacardic acid analogues possessing different side chains viz. phenolic, branched, and alicyclic were synthesized and their antibacterial activity tested against methicillin-resistant *Staphylococcus aureus* (MRSA). The maximum activity against this bacterium occurred with the branched side-chain analogue, 6-(4′,8′-dimethylnonyl)salicylic acid, and the alicyclic side-chain analogue, 6-cyclododecylmethyl salicylic acid, with the minimum inhibitory concentration (MIC) of 0.39 μg/mL, respectively. This activity was superior to that of the most potent antibacterial anacardic acid isolated from the cashew *Anacardium occidentale* (Anacardiaceae), apple and nut, that is, the 6-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]salicylic acid.

1. Introduction

In our previous reports on structure–antimicrobial activity relationship (SAR) studies on a series of aliphatic alcohols, their maximum antimicrobial activity appeared to be an interactive function of the hydrophobic alkyl (tail) chain length and the hydrophilic hydroxyl group (head). It was proposed that the hydrophilic head moiety binds with an intermolecular hydrogen bond like a 'hook' attaching itself to a hydrophilic portion of the membrane, after which the hydrophobic tail portion of the molecule is then able to enter into the membrane lipid bilayer. As a result, disorder in the fluid bilayer of the membrane is created and this conclusion may be explained by the alcohols' non-ionic surfactant properties as well as by their non-specificity in the activity. It also appears that biophysical processes are a major contributor to the antimicrobial activity of amphipathic alkanols.[1,2] The hydrophilic hydroxyl group can be replaced by any other hydrophilic groups as long as there is a balance in the 'head and tail' structure. Hence, various additional biological activities can be introduced in these scaffolds mainly by selecting appropriate head portions.[3]

Staphylococcus aureus is one of the main bacteria that cause suppuration, food poisoning, and toxic shock syndrome[4] and is thus the focus of this work. Strains of methicillin-resistant *S. aureus* (MRSA) are known to be resistant to many antibiotics and currently represent a serious problem to hospitalized patients as well as their caretakers.[5] Among the important biological activities known about anacardic acids is their antibacterial activity and this has been most extensively studied.[1,2,6–10] In addition, due to its adaptability, *S. aureus* can easily develop resistance to the commonly used antibiotics. This resistance involves both an enzymatic inactivation as well as the presence of an altered penicillin binding protein in the resistant bacteria.[11] Such resistance genes are then often transferred to other bacteria by a variety of gene transfer mechanisms[12] which exacerbates the problem further. It is thus imperative that the search for new anti-MRSA agents continues since it is crucial to the future maintenance of public health.[13] Additionally, there is a great need for more effective antibacterial agents having new modes of action and consequently the ideal antimicrobial agent should best be produced by rational design.[14] Antibacterial agents that primarily act as surface-active ones viz. surfactants may have the potential of addressing this need, since they target the extracytoplasmic region and thus do not need to enter

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the cell, thereby avoiding most cellular pump-based resistance mechanisms. Possible structural variations in the head and tail moieties of the anacardic acid scaffold suggest that optimization of activity is possible through a process of selective synthetic design. To date, only the non-branched aliphatic chains having various lengths viz. (C_5, C_8, C_{10}, C_{12}, and C_{20}) have been synthesized.[7] It is thus conceivable that optimization of more specific activity is a strong possibility through the synthetic approach in which cases the molecular dimensions, together with the lipophilicity, would have a critical impact on the biologically active profile of these molecules.

2. Results and discussion

The anacardic acids viz. 6-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]salicylic acid (**1**) (see Fig. 1 for structures), will be referred to as anacardic acid ($C_{15:3}$) for simplicity hereafter; and 6-[8′(*Z*),11′(*Z*)-pentadecadienyl]salicylic acid (**2**), as anacardic acid ($C_{15:2}$), and 6-[8′(*Z*)-pentadecenyl]salicylic acid (**3**) as anacardic acid ($C_{15:1}$) are all isolated from the cashew apple, *Anacardium occidentale* (Anacardiaceae) and are well known to exhibit potent antibacterial activity against Gram-positive bacteria including MRSA strains.[15,16] Since these anacardic acids are salicylic acid derivatives having a C-6 alk(en)yl side chain, their inhibitory activity was also compared with that of salicylic acid.[7] In previous papers, anacardic acid ($C_{15:3}$) (**1**) was described to exhibit bactericidal activity against *S. aureus* at any growth stage, and additionally even when cell division was inhibited by chloramphenicol.[9] In general anacardic acids are amphipathic molecules, which implies that their hydrophobic properties dominate the properties of the molecule. This finding is consistent with the proposed mechanism of their antibacterial activity which involves disordering of the membrane. However, the specific site of action for this disordering effect is less clear. The primary response for the antibacterial activity of anacardic acids arises from their biophysical disruption of the membrane, indicating that the alk(en)yl side chain is an important scaffold to elicit this activity. Furthermore, anacardic acids were also reported to inhibit the oxidation of NADH oxidase by a membrane fraction prepared from *Micrococcus luteus* cells, indicating that anacardic acids also inhibit respiratory chain enzyme activity to demonstrate their wider activity profile. However, although this biochemical mechanism is not a primary contributor to elicit their antibacterial activity[2], it does, however, not exclude the possibility that anacardic acids first act as surfactants after which biochemical processes become involved, a factor which needs to be born in mind when drawing conclusions about possible mechanisms. The antibacterial activity of anacardic acids against *S. aureus* was previously found to be proportional to the degree of the side-chain unsaturation.[6,17] However, a recent study indicates that the double bond in the C-6 side chain is not essential in eliciting the antibacterial activity but is rather associated with increasing the activity.[2] In addition, anacardic acid ($C_{15:0}$) (**4**) has previously been reported to show high selectivity toward Fe^{2+} and Cu^{2+}.[18] This finding implies that chelation might also play a role in the antimicrobial activity of anacardic acids by reducing their bioavailability for bacteria.[19] Evidence currently available tends to support this possibility since anacardic acid ($C_{15:0}$) (**4**) did not show any antibacterial activity against *S. aureus* up to 800 μg/mL. On the other hand, it has also been shown that activity is inversely proportional to the length of the C-6 side chain since the hydrophobicity of molecules is well known to play a critical role in biological activities.[20] However, the rationale for this, especially the role of the hydrophobic portion, is still poorly understood, since this area of study has received scant attention. Although these anacardic acids, vide infra, are available in quantities, they are rather unstable for practical applications due to their side-chain unsaturation.

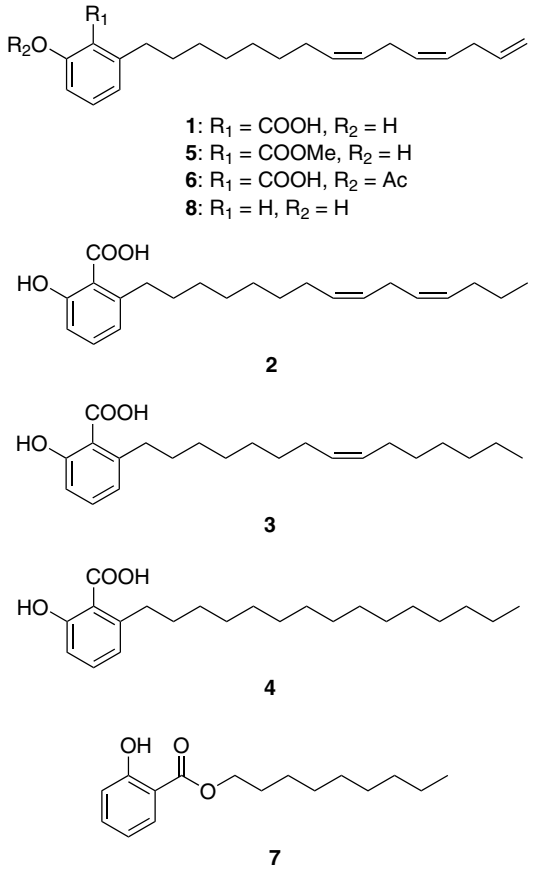


Figure 1. Structures of anacardic acid and its related compounds.

Neither methyl ester (**5**) nor acetate (**6**) of anacardic acid ($C_{15:3}$) exhibited any antibacterial activity against *S. aureus* up to 200 μg/mL,[21] indicating that the free salicylic acid moiety is an essential element to elicit the antibacterial activity. In addition, anacardic acids are known to exhibit potent antibacterial activity against MRSA strains but salicylic acid itself has little effect on these strains which leads to the conclusion that the hydrophobic alk(en)yl side chain is undoubtedly associated with the antibacterial activity. Hence, it may not be far fetched to assume that side-chain alkyl salicylates would demonstrate varying degrees of antibacterial activity

against *S. aureus*. To this end, nonyl salicylate (**7**) lacking the C-6 alkyl side chain was synthesized and tested for antibacterial activity against *S. aureus*. No antibacterial activity was found again supporting the contention that the 6-alk(en)yl salicylic acid molecular scaffold is a prerequisite to elicit the activity. This conclusion is further supported by the observation that cardanol (**8**), an artifact of the corresponding anacardic acid ($C_{15:3}$) obtained by pyrolysis, did not show any antibacterial activity against *S. aureus* up to 200 μg/mL.[22] In addition to molecules having the anacardic acid scaffold, it should be further noted that the degree of antimicrobial activity depends both on the hydrophobic tail portion as well as the microorganisms being tested.[7] Abundant literature examples have shown that a small change in the chemical structure of molecules affects their biological activities to a large extent. This prompted us to synthesize a series of anacardic acid analogues possessing different side chains viz. phenolic, branched, and alicyclic in order to gain new insights into their antibacterial activity on a molecular basis. The introduction of branching or unsaturation into the hydrophobic moiety is known to increase the solubility of the surfactant in water.[23] If the hydrophobic portion of the molecule enters into the membrane lipid bilayer and creates disorder in the fluid bilayer, then it stands to reason that by increasing the volume of the hydrophobic portion through synthetic modification, the resulting analogue may demonstrate an enhancement in its activity.

In addition to their antibacterial activities, anacardic acids are also known to act as antioxidants in a variety of ways including inhibition of various prooxidant enzymes involved in the production of the reactive oxygen species and additionally chelate divalent metal ions such as Fe^{2+} or Cu^{2+}, but do not quench reactive oxygen species. Recently, their lipoxygenase inhibitory activity has been reported.[24] Lipoxygenases (EC 1.13.11.12) are suggested to be involved in the early event of atherosclerosis by inducing plasma low-density lipoprotein (LDL) oxidation.[25,26] For this and other reasons, the molecular design of our compounds was dictated to by keeping in mind these various biological activities. Second, we reasoned that since phenolic compounds are generally considered to possess antibacterial activity and are used as domestic disinfectants, attaching them to the side chain of the anacardic acid nucleus would be a valid area to investigate. Third, it is also well known that the hydrophobic tail chain length and degree of unsaturation plays a role in the activity of anacardic acids[1,2] and that some initial attempts to quantify this have been made.[7] Consequently, it was reasoned that this aspect needed to be broadened and thus we included some analogues with variations of chain length and branching to comparatively evaluate these to known anacardic acids. Finally, the molecular volume of the hydrophobic side chains at C-6 in anacardic acids has been speculated to play a role in increasing the antimicrobial activity[7] and this prompted us to synthesize a new series of anacardic acid analogues having saturated hydrophobic carbocyclic ring systems of differing size to thus represent increasing molecular volumes at C-6 and evaluate these on a comparative basis (Fig. 2).

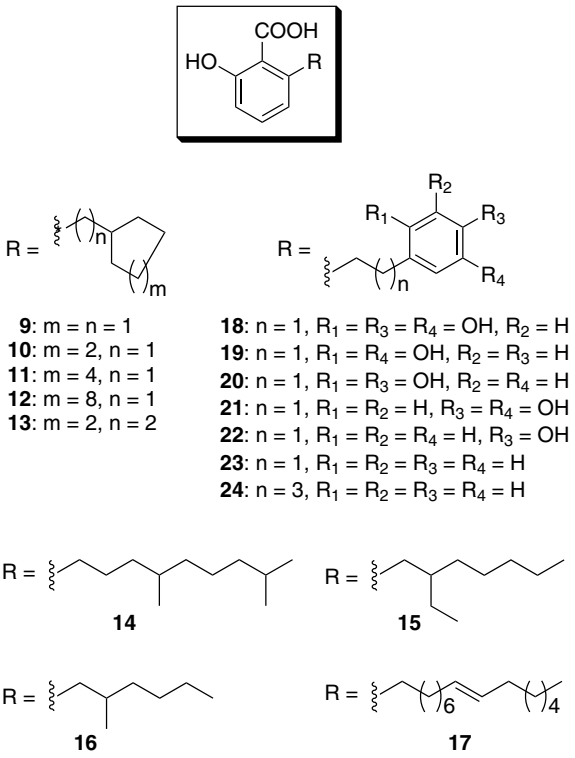
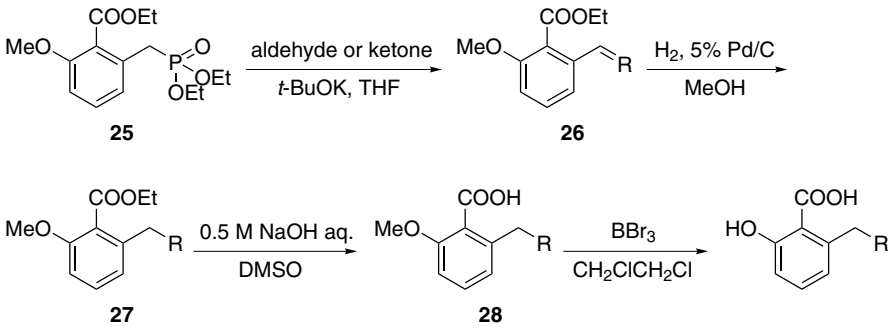


Figure 2. Structures of anacardic acid derivatives.

The synthesis of non-branched 6-alkylsalicylic acids has previously been described[7,27] and thus 15 new anacardic acid analogues (**9**–**24**) were synthesized by a standard protocol developed in our laboratories and shown in Scheme 1.[28–30] In brief, phosphonate ester **25** and commercially available aldehydes were condensed under basic Wittig type conditions to afford the corresponding *trans* olefins **26** in average yields of 60%. Catalytic hydrogenation of these olefins afforded the saturated aryl esters **27** (85%) which were in turn hydrolyzed in strongly basic dimethylsulfoxide at 110 °C to produce the corresponding aryl carboxylic acids **28** (85%). Demethylation of the aryl methoxy group was accomplished using boron tribromide in 1,2-dichloroethane at low temperature to afford the anacardic acid analogues (70%). Thus the average transformation of phosphonate ester **25** into the anacardic acid analogues was 26% for the four steps.

The synthesized compounds were tested for their antibacterial activity against *S. aureus* ATCC 33592 (MRSA) using a 2-fold serial broth dilution method. The highest concentration tested was 200 μg/mL because of the solubility limitation of some samples in the water based medium. Results are listed in Table 1 and the data obtained were compared with those of anacardic acid ($C_{15:3}$) (**1**).[2,10] All synthesized compounds were also tested for their antibacterial activity against *Escherichia coli* ATCC 9637, but none of them exhibited any activity up to 200 μg/mL.

The alicyclic anacardic acid analogues **9**–**13** showed antibacterial activity against *S. aureus* with MICs ranging from 0.39 to 100 μg/mL. Among these compounds,



Scheme 1. Syntheses of anacardic acid derivatives.

Table 1. Minimal inhibitory concentrations of natural anacardic acids and their analogues for methicillin-resistant *Staphylococcus aureus* ATCC 33592

Compounds tested	MIC (μg/mL)	log *P*
6-[8′(*Z*),11′(*Z*),14′-Pentadecatrienyl]salicylic acid (**1**)	6.25[a]	6.62
6-[8′(*Z*),11′(*Z*)-Pentadecadienyl]salicylic acid (**2**)	12.5[a]	6.89
6-[8′(*Z*)-Pentadecenyl]salicylic acid (**3**)	100[a]	7.21
6-(Pentadecenyl)salicylic acid (**4**)	>800[a]	7.53
6-[8′(*Z*),11′(*Z*),14′-Pentadecatrienyl]salicylic acid methyl ester (**5**)	>800	6.89
Acetyl 6-[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]salicylic acid (**6**)	>800	6.60
6-Cyclopentylmethyl salicylic acid (**9**)	100	3.19
6-Cyclohexylmethyl salicylic acid (**10**)	25	3.61
6-Cyclooctylmethyl salicylic acid (**11**)	12.5	4.44
6-Cyclododecylmethyl salicylic acid (**12**)	0.39	6.11
6-Cyclohexylethyl salicylic acid (**13**)	12.5	4.02
6-(4′,8′-Dimethylnonyl)salicylic acid (**14**)	0.39	5.69
6-(2′-Ethylheptyl)salicylic acid (**15**)	6.25	4.52
6-(2′-Methylhexyl)salicylic acid (**16**)	50	3.69
6-(8′*E*-Pentadecaenyl)salicylic acid (**17**)	6.25	7.21
6-[2′-(2″,4″,5″-Trihydroxyphenyl)ethyl]salicylic acid (**18**)	>200	2.54
6-[2′-(2″,5″-Dihydroxyphenyl)ethyl]salicylic acid (**19**)	100	2.93
6-[2′-(2″,4″-Dihydroxyphenyl)ethyl]salicylic acid (**20**)	>200	2.93
6-[2′-(3″,4″-Dihydroxyphenyl)ethyl]salicylic acid (**21**)	100	2.93
6-[2′-(4″-Hydroxyphenyl)ethyl]salicylic acid (**22**)	50	3.32
6-(2′-Phenylethyl)salicylic acid (**23**)	100	3.71
6-(4′-Phenylbutyl)salicylic acid (**24**)	25	4.55

[a] The data were extracted from previous papers.[2,10]

the maximum activity was found for 6-cyclododecylmethyl salicylic acid (**12**) with an MIC of 0.39 μg/mL. In contrast, 6-cyclopentylmethyl salicylic acid (**9**) representing the smallest side-chain ring molecule in the series was the least active analogue with an MIC of 100 μg/mL. In general the activity of compounds **9**–**13** increased in direct proportion to the number of C-atoms in the cyclic ring at C-6.

The activity of **12** increased 16 times compared to that of anacardic acid ($C_{15:3}$) (**1**). Compound **10** was 50% less active than **13**, with the essential difference between them being an additional C-atom separating the phenyl and cyclohexyl rings. The MIC of 6-cyclohexylethyl salicylic acid **13** was 12.5 μg/mL for MRSA. 6-Cyclooctylmethyl salicylic acid **11** exhibited stronger activity than those of cyclopentylmethyl and cyclohexylmethyl salicylic acid indicating that the molecular volume of the side chain is an important contributing factor to improved activity a fact very clearly demonstrated by analogue **12** having the dodecyl ring. The MIC of **11** for MRSA was 12.5 μg/mL.

In the aliphatic side chain series, the maximum activity of C-6 branched side-chain anacardic acid analogues against MRSA was found with 6-(4′,8′-dimethylnonyl)salicylic acid **14** with an MIC of 0.39 μg/mL. The activity of **14** against MRSA was also 16 times stronger than that of natural anacardic acid ($C_{15:3}$) (**1**). With an MIC of 50 μg/mL, 6-(2′-methylhexyl)salicylic acid **16** was the least active branched side-chain anacardic acid analogue and also incidentally represented the shortest side chain in this series of analogues. The MIC of analogue **15** with a C_9H_{19} branched side chain was 6.25 μg/mL. It would thus appear that the more hydrophobic the C-6 side chains of anacardic acids are, the more readily they should be able to disrupt membrane integrity.

When tested against MRSA compound **15** exhibited the same antibacterial activity as the natural anacardic acid ($C_{15:3}$) (**1**) with an MIC of 6.25 μg/mL. It seems that a branched side chain increases antibacterial activity of 6-alkylsalicylic acids and that the presence of a double bond in the C-6 hydrocarbon side chain may not

contribute all that significantly to the activity of these types of analogues.

The anacardic acid analogues **18**–**24** having phenolic moieties in their side chains at C-6 exhibited antibacterial activities in which the MICs ranged between 25 and >200 μg/mL. The maximum activity occurred with 6-(4′-phenylbutyl)salicylic acid (**24**) that had an MIC of 25 μg/mL which is superior to that of 6-phenylethylsalicylic acid (**23**) that had an MIC of 100 μg/mL for MRSA. It thus seems that both the length of the linker alkyl side chain C4 in (**24**) versus C2 in (**23**) and the presence or absence of hydroxyl groups in the aryl ring viz. 4-OH in (**22**) versus 4-H in (**23**) may also play an important role in increasing the activity of these molecules. However, the absolute activity of **24** was no better than that of 6-[8′(*Z*),11(*Z*),14′-pentadecatrienyl]salicylic acid (**1**) that had an MIC 6.25 μg/mL.

One of the more important observations made from this work was that the antibacterial activity was generally lower for those anacardic acid analogues having a phenolic and not an alkyl side chain at C-6. For example, the MICs of compounds **18**–**22** that contain between one to three hydroxyl groups on the aromatic ring of the side chain varied between 50 and >200 μg/mL for MRSA.

It is possible that the presence of hydrophilic groups on both phenyl rings separately and collectively in the molecule may lower the antibacterial activity against MRSA.[1] On the other hand, the MICs of the compounds **18**–**21** suggest that the activity against *S. aureus* is only affected by the number and position of the hydroxyl groups on the phenyl ring of the side chain. For instance, the activity of compound **18** (MIC >200 μg/mL) which contains three hydroxyl groups was lower than that of either **19** or **21** (MIC's of 100 μg/mL) both of which possess two hydroxyl groups on the side-chain phenyl ring. On the other hand, compound **20** which also possesses two hydroxyl groups on the phenyl ring but at different positions, that is, a different regioisomer did not show any activity against MRSA up to 200 μg/mL. When tested against MRSA the MIC of **18** was comparable with that of **20**. It is thus clear that in these cases the actual substitution pattern of the hydroxyl groups on the phenyl rings has a role to play in the activity. It is too early to speculate on the possible reasons for this with the information at hand.

Anacardic acids are known to act as multifunctional agents with the two most important being antibacterial and antioxidant.[24,31,32] All the compounds tested are assumed to possess chelation ability since this activity originates from the salicylic acid moiety (the functional unit in anacardic acid).[33,34] In the cases of compounds **18**–**24**, none of these analogues exhibited notable antibacterial activity, but some showed good antioxidant activity. For example, **18** and **19** were found to scavenge DPPH and **19** has been described to demonstrate weak inhibitory activity of potato lipoxygenase.[18]

3. Conclusion

Since the head portion of the anacardic acids studied in this series is the same, data are interpreted to signify that changes in the hydrophobic tail portions may be correlated and are indeed responsible for the specific activity. As far as anacardic acids (**1**–**4**) are concerned, their antibacterial activity can be optimized by selecting a suitable side chain length to give the appropriate partition coefficient (log *P*) as a standard. Our current study has demonstrated that other factors viz. branching and volumetric size introduced into the hydrophobic moiety which is known to increase the solubility of the molecules in water and in this way cause looser packing of the surfactant molecules at the interface is also a major contributing factor associated with the activity.[23] The surfactant molecules showed significant toxicity at higher concentrations. It is thus a strong possibility that anacardic acids and their derivatives might be non-specific cytotoxic agents. However, it has been demonstrated that anacardic acid ($C_{15:3}$) does not induce chromosome damage in vivo,[35] indicating that their analogues may thus only possess low genotoxic properties. In view of the increasing importance of controlling specific bacteria such as MRSA, studies directed at both the branched and alicyclic side chain anacardic acid analogues have demonstrated that they do have the potential to pave the way for a new generation of drugs with anti-MRSA activity.

4. Experimental

4.1. Chemicals

Anacardic acids and their analogues were available from previous works,[1,7,15,27] and synthesis of 6-(8′*E*-pentadecaenyl)salicylic acid **17** was previously described.[28–30] Salicylic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). *N*,*N*-Dimethyl formamide (DMF) was obtained from EM Science (Gibbstown, NJ). Other reagents were purchased from commercial suppliers and used as received, unless otherwise noted.

4.2. Synthesis

The synthetic work was previously published.[28–30] In brief, to a stirred solution of phosphonate ester **25**[7] (2.0 g; 6.6 mM) in dry THF (20 mL) at 0 °C was added a solution of potassium *t*-butoxide (1.25 g; 11.2 mM) in THF (10 mL) under an atmosphere of nitrogen. The resulting mixture was stirred at 0 °C for a further 30 min after which a solution of the aldehyde (6.6 mM) in THF (15 mL) was added. Stirring was continued at 0 °C for a further 10 min and the solution then allowed to warm up to 25 °C and stirred for another 12 h. The reaction mixture was quenched with saturated aqueous ammonium chloride (100 mL) and extracted with ether to afford a residue that was purified by column chromatography using ethyl acetate–hexane (1/4–2/3) as eluent to yield the *trans* olefins **26**. Catalytic hydrogenation of the olefins **26** over Pd(0) (5% Pd/C)

yielded the corresponding saturated aryl esters **27**. A solution of the ester **27** (2 mM) in DMSO (5 mL) was treated with aqueous sodium hydroxide (5 mL of a 0.5-M solution, 2.5 mM) and stirred in an oil bath at 110 °C for 12 h after which the cooled solution was acidified (litmus) with aqueous hydrogen chloride (1 M) and then diluted with water to a volume of 20 mL and then extracted with ethyl acetate. The residue obtained was recrystallized from ethyl acetate–hexane to afford the corresponding methoxy benzoic acids **28**. Finally, to a stirred solution of the methoxy benzoic acids **28** (1 mM) in 1,2-dichloroethane (10 mL) at between 25 and −78 °C was added boron tribromide (1.1 mM per methoxy group present) under nitrogen and stirring was continued for 3 h. The temperature of the reaction mixture was allowed to rise to 25 °C after which water (100 mL) was added and the solution acidified (litmus) by the addition of aqueous hydrogen chloride (1 M). Extraction with ethyl acetate afforded the anacardic acids.

4.3. Test organism and medium

Methicillin resistant *S. aureus* ATCC 33591 and *E. coli* ATCC 9637 were obtained from American Type Culture Collection (Manassas, VA). Antibacterial assay was performed using NYG broth, consisting of 0.8% nutrient broth, 0.5% yeast extract, and 0.1% glucose. Nutrient broth and yeast extract were purchased from BD (Franklin Lakes, NJ).

4.4. MIC determination

The MICs were determined by the 2-fold broth dilution method as previously described.[1,9] 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, which consisted of 0.8% nutrient broth, 0.5% yeast extract, and 0.1% glucose. These were inoculated with 30 μL of an overnight culture of the test bacterium. After incubation of the cultures at 37 °C for 48 h, the MIC was determined as the lowest concentration of the test compound that demonstrated no visible growth. Because of solubility limitation of the samples in the water based medium, the highest concentration tested in the assay was 200 μg/mL, unless otherwise specified. The MIC of each compound was determined at least in triplicate on separate occasions.

4.5. Log *P* calculation

Log *P* values were calculated by Chem Draw Pro version 4.5 developed by Cambridge Soft Co. (Cambridge, MA) using Crippen's fragmentation.[36]

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