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Short communication

Design and evaluation of anacardic acid derivatives as anticavity agents

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Abstract

On the basis of antibacterial anacardic acids, 6-pentadecenylsalicylic acids, isolated from the cashew apple, *Anacardium occidentale* L. (Anacardiaceae), a series of 6-alk(en)ylsalicylic acids were synthesized and tested for their antibacterial activity against *Streptococcus mutans* ATCC 25175. Among them, 6-(4′,8′-dimethylnonyl)salicylic acid was found to exhibit the most potent antibacterial activity against this cariogenic bacterium with the minimum inhibition concentration (MIC) of 0.78 μg/ml.

Keywords: Anacardic acid; *Streptococcus mutans*; Antibacterial activity; 6-(4′,8′-Dimethylnonyl)salicylic acid

1. Introduction

Dental caries is one of the most ubiquitous infectious disease in developed countries. Interactive elements, including nutritional intake and presence of cariogenic microflora, are contributors to this disease. Many recent studies have concluded that *Streptococcus mutans* is the primary bacteria causing dental caries [1,2]. This bacterium adheres firmly to smooth tooth surfaces and facilitates the accumulation of other oral microorganisms. Predominant in plaque, *S. mutans* and other accumulated microorganisms generate organic acids, primarily lactic acid, that gradually destroy the enamel surface thereby creating an opening susceptible to further bacterial degradation and ultimately forming a cavity [1,3]. Theoretically, dental caries should be preventable by eliminating *S. mutans*. The difficulties associated with eliminating this cariogenic bacterium by means of chemical methods are easily recognized [4]. Among them, safety of the chemicals seems to be the most important, since *S. mutans* resides in the mouth.

Anacardic acids; 6[8′(*Z*),11′(*Z*),14′-pentadecatrienyl]salicylic acid (**1**), 6[8′(*Z*),11′(*Z*)-pentadecadienyl]salicylic acid (**2**), and 6[8′(*Z*)-pentadecenyl]salicylic acid (**3**), were previously isolated from the cashew apple *Anacardium occidentale* L. (Anacardiaceae) [5,6], and for the purposes of this paper are referred to as anacardic acid ($C_{15:3}$), anacardic acid ($C_{15:2}$) and anacardic acid ($C_{15:1}$) for simplicity, respectively (see Fig. 1 for structures). Their diverse biological activities have been reported which include their potent antibacterial activity against the Gram-positive bacteria viz. *S. mutans* [7–11]. These anacardic acids, which are contained in a regularly imbibed beverage known as cashew apple juice, may be considered then as beneficial anticavity agents. However, the anacardic acids are rather unstable for practical applications because of their side chain unsaturation. This prompted us to synthesize a series of anacardic acid analogues possessing different side chains viz. phenolic, branched and alicyclic by essentially a four-step protocol [12,13].

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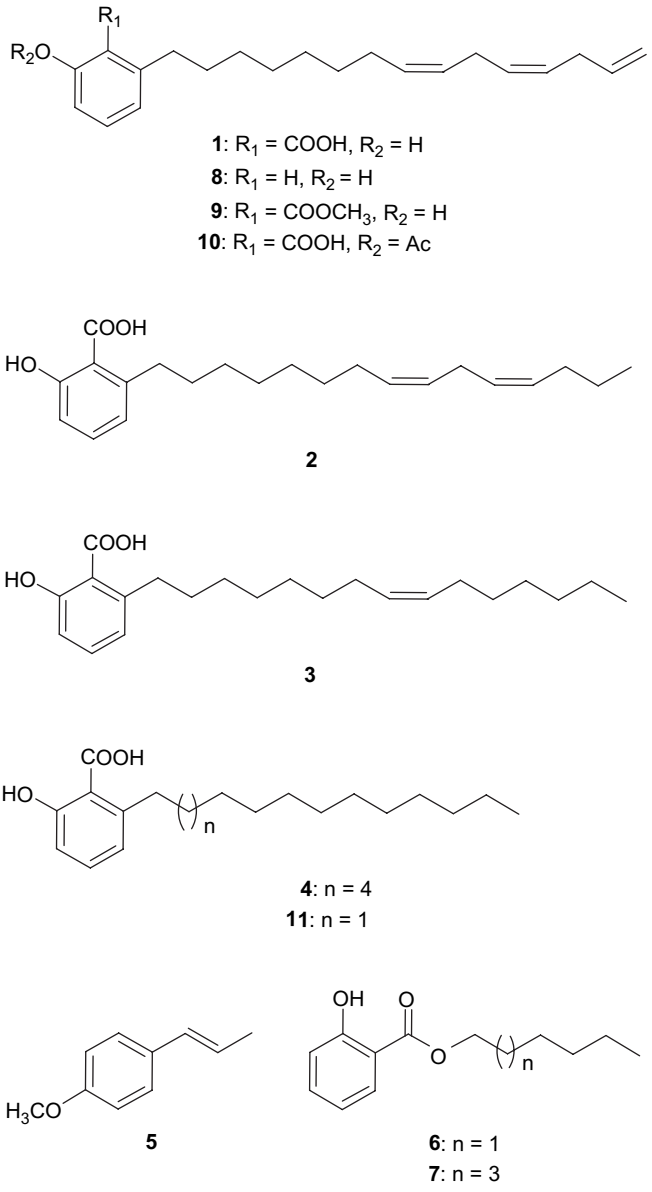


Fig. 1. Structures of natural occurring anacardic acids **1**–**3** and their related compounds **4**–**11**.

2. Results and discussion

In previous studies on the antibacterial activity of a number of anacardic acids **1**–**3** against *S. mutans* ATCC 25175, the emphasis was placed on anacardic acid ($C_{15:3}$) (**1**) because this compound showed the most potent bactericidal activity with the minimum bactericidal concentration (MBC) of 6.25 μg/ml (Table 1). The bactericidal activity was confirmed by time-kill curve experiments [7,10]. The antimicrobial activity of anacardic acids was proportional to the degree of the side chain unsaturation and can be summarized in the following decreasing order; $C_{15:3} > C_{15:2} > C_{15:1}$. However, a recent study indicates that the double bond is not essential in eliciting the antibacterial activity but is more likely to be associated with increasing the inherent activity of the compound [14]. Interestingly, anacardic acid ($C_{15:2}$) (**2**) demonstrated an almost

Table 1
Minimal inhibitory concentrations of anacardic acids against *Streptococcus mutans* ATCC 25175

Compounds tested	MIC and MBC (μg/ml)[a]	log *P*
Anacardic acid ($C_{15:3}$) (**1**)	1.56 (6.25)	6.62
Anacardic acid ($C_{15:2}$) (**2**)	3.13 (3.13)	6.89
Anacardic acid ($C_{15:1}$) (**3**)	6.25 (6.25)	7.21
Anacardic acid ($C_{15:0}$) (**4**)	>800 (−)	7.53
Anacardic acid ($C_{12:0}$) (**11**)	1.56 (6.25)	6.28

[a] The data were extracted from our previous papers [7,8,10].

equally as potent activity as anacardic acid ($C_{15:3}$) against *S. mutans*, whereas anacardic acid ($C_{15:0}$) (**4**) did not show any activity up to 800 μg/ml [7]. Anacardic acids are salicylic acid derivatives with a non-isoprenoid alkenyl side chain and therefore the differences observed in the activity should be interpreted to imply that changes in the hydrophobic side chain tail portions are the major influential dictators to the activity. Anacardic acids **1**–**3** can be isolated in fair quantities from cashew nut shell liquid (CNSL) [5] which in turn, is available in large quantities as agricultural waste. However, the following two disadvantages of anacardic acids need to be considered.

Firstly, the observed bactericidal activity of anacardic acid ($C_{15:3}$) against *S. mutans* may not be potent enough for practical applications. This is always a dilemma when the biological activities of phytochemicals, particularly their antimicrobial activity, are considered. Hence, studies to enhance their activity are always considered to be of the utmost importance and one of the more promising strategies to enhance their activity consists of combining two or more phytochemicals in their application. Thus, combining two or more compounds might, in general, be superior to the use of a single antimicrobial compound in order to decrease the likelihood for the development of resistance mechanisms by the microorganisms, in addition to enhancing and/or broadening their total biological activity spectrum. Antibacterial agents used against *S. mutans* were previously isolated from edible plants [7,15]. The bactericidal activity of the sweet tasting anethole (**5**) against this cariogenic bacterium was previously confirmed by the time-kill curve experiment [7]. However, bactericidal activity of anacardic acid ($C_{15:3}$) against *S. mutans* was enhanced 8-fold when in combination with a sublethal amount (equivalent to 1/2 MBC) of anethole. Thus, in this way, the MBC of anacardic acid ($C_{15:3}$) was lowered from 6.25 to 0.78 μg/ml in combination with 200 μg/ml of anethole.

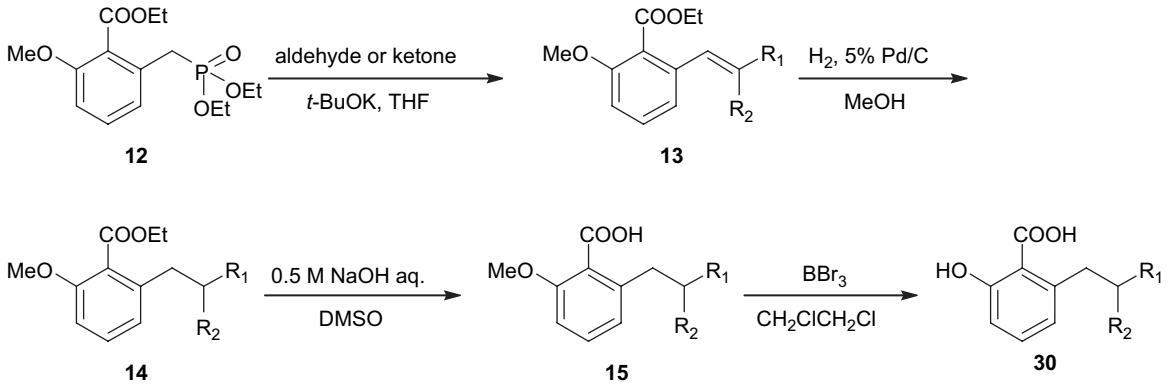
Secondly, and more importantly, anacardic acid ($C_{15:3}$) is rather unstable for practical applications because of its side chain unsaturation. The inter-relationship between the head and tail moieties of anacardic acids isolated from the cashew apple suggests that optimization of activity is a real possibility through the synthetic approach. Since anacardic acids **1**–**4** are salicylic acid derivatives with a pentadec(en)yl side chain, it was reasoned that alkyl salicylates may show similar antibacterial activity. Hence, both hexyl 2-hydroxybenzoate (**6**) and nonyl 2-hydroxybenzoate (**7**) were synthesized and tested to determine whether they exhibit antibacterial activity against

S. mutans. It was, however, demonstrated that, neither salicylate showed this activity, which indicated that the 6-alkylsalicylic acid scaffold structure is a prerequisite to elicit this particular antibacterial activity against *S. mutans*. This suggestion can be indirectly supported by the observation that cardanol ($C_{15:3}$) (**8**) [8], an artifact of the corresponding anacardic acid ($C_{15:3}$) obtained by pyrolysis [6], did not show any noticeable antibacterial activity against *S. mutans*. In addition, the previous observation that neither methyl ether **9** nor acetate **10** of anacardic acid ($C_{15:3}$) exhibited any noticeable antibacterial activity against *S. mutans* lent further support for this conclusion.

In general it is observed that antimicrobial activity is inversely proportional to the length of the C-6 side chain and that at a particular critical length it reaches a maximum after which activity greatly diminishes to finally become inactive. If only 6-alkylsalicylic acids are compared, their calculated partition coefficient (calculated log *P*) values seem to be in proportion to their antibacterial activity and the maximum activity against *S. mutans* seems to be at a value of log *P* of around 6 [16]. Hence, a series of anacardic acids possessing different lengths of the C-6 side chain were synthesized. Among these compounds, anacardic acid ($C_{12:0}$) (**11**) exhibited the most potent bactericidal activity against *S. mutans* with an MBC of 6.25 μg/ml [8], while anacardic acid ($C_{15:0}$) did not show any activity up to 800 μg/ml. It was important to include anacardic acid ($C_{15:0}$) since it can easily be obtained by hydrogenation over 5% Pd–C of the anacardic acid-containing fraction of CNSL. It should be noted, however, that although anacardic acid ($C_{15:0}$) was not effective against *S. mutans* it nevertheless exhibited potent antibacterial activity against *Propionibacterium acnes* ATCC 11827 with a minimum inhibitory concentration (MIC) of 0.78 μg/ml [17]. From the foregoing results it appears that the MBC of 6.25 μg/ml of anacardic acid ($C_{12:0}$) seems to be a maximum and thus optimization of activity through the synthetic approach would certainly offer an obvious solution for a more comprehensive evaluation. However, in the initial optimization investigation through the synthetic approach, only analogues containing the non-branched aliphatic chains having various lengths were synthesized [7,18]. This suggested that further optimization is indeed still achievable and hence, a series of anacardic acid analogues possessing different C-6 side chains viz. phenolic, branched and alicyclic were synthesized [12,13]. In brief, phosphonate ester **12** and commercially available aldehydes were condensed under basic Wittig type conditions to afford the corresponding *trans* olefins **13** in average yields of 60%. Catalytic hydrogenation of these olefins afforded the saturated aryl esters **14** (85%) which were hydrolysed by sodium hydroxide in dimethyl sulfoxide (DMSO) at 110 °C to produce the corresponding aryl carboxylic acids **15** (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 **12** into the anacardic acid analogues was 26% for the 4 steps [12,13] (Scheme 1).

The three structurally different types of anacardic acid analogues that were synthesized totaling sixteen were subsequently tested for their antibacterial activity against *S. mutans* ATCC 25175 using a 2-fold serial broth dilution method. The highest concentration tested was 200 μg/ml because of a solubility limitation of some samples in the water based medium. The results are listed in Table 2. The data obtained were compared with that of anacardic acid ($C_{15:3}$) which is usually taken as the most active member of the naturally occurring anacardic acids. All the 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 synthetic anacardic acid analogues **16–22** representing the hydroxyl- and non-hydroxyphenyl analogues at C-6 exhibited a varied activity against *S. mutans* ATCC 25175 in which the MIC ranged between 6.25 and >200 μg/ml. The maximum activity against *S. mutans* occurred for analogue **22** which had an MIC of 6.25 μg/ml. This activity of analogue **22** was 8-fold superior to that of 6-phenylethylsalicylic acid **21** which had an MIC of 50 μg/ml. It consequently appears that the length of the linker alkyl side chain plays an important role in increasing antibacterial activity. However, the activity of analogue **22** against *S. mutans* was not superior to that of anacardic acid ($C_{15:3}$) which has an MIC of 1.56 μg/ml. The most important observation in this series was that the antibacterial activity was much lower for the anacardic acid analogues having a phenolic side chain. For example the MICs of the compounds **16–22** that contain one to three hydroxyl groups



Scheme 1. Synthesis of anacardic acid derivatives **12–30**.

Table 2
Minimal inhibitory concentrations of C-6 phenolic, alicyclic and branched anacardic acid analogues against *Streptococcus mutans* ATCC 25175

Compounds tested	MIC (μg/ml)	log *P*
6-[2′-(2″,4″,5″-Trihydroxyphenyl)ethyl]salicylic acid (**16**)	200	2.54
6-[2′-(2″,5″-Trihydroxyphenyl)ethyl]salicylic acid (**17**)	200	2.93
6-[2′-(2″,4″-Trihydroxyphenyl)ethyl]salicylic acid (**18**)	>200	2.93
6-[2′-(3″,4″-Trihydroxyphenyl)ethyl]salicylic acid (**19**)	200	2.93
6-[2′-(4″-Hydroxyphenyl)ethyl]salicylic acid (**20**)	100	3.32
6-(2′-Phenylethyl)salicylic acid (**21**)	50	3.71
6-(4′-Phenylbutyl)salicylic acid (**22**)	6.25	4.55
6-Cyclopentylmethylsalicylic acid (**23**)	25	3.19
6-Cyclohexylmethylsalicylic acid (**24**)	25	3.61
6-Cyclooctylmethylsalicylic acid (**25**)	6.25	4.44
6-Cyclododecylmethylsalicylic acid (**26**)	1.56	6.11
6-Cyclohexylethylsalicylic acid (**27**)	3.13	4.02
6-(4′,8′-Dimethylnonyl)salicylic acid (**28**)	0.78	5.69
6-(2′-Ethylheptyl)salicylic acid (**29**)	3.13	4.52
6-(2′-Methylhexyl)salicylic acid (**30**)	12.5	3.69

on the aromatic ring of the side chain varied between 100–200 μg/ml for *S. mutans.*

Most probably, the presence of hydrophilic groups on both phenyl rings of the molecule, referred to as the head and tail structure, decreases the overall antibacterial activity against *S. mutans* [9]. On the one hand the MICs of the compounds **16–19** suggest that the activity against *S. mutans* is not affected by either the number or position of the hydroxyl group(s) on the phenyl ring of the side chain. Thus, the activity of compound **16**, which contains three hydroxyl groups, was comparable with that of the **17** and **19** both possessing two hydroxyl groups on the phenyl ring of the side chain. The MIC of **17** and **19** for *S. mutans* was 200 μg/ml. On the other hand, compound **18** did not show any activity against this cariogenic bacterium up to 200 μg/ml and this is due to the particular oxygenation pattern on the phenyl ring.

The alicyclic anacardic acid analogues **23–27** showed far more promising activity against *S. mutans* with MICs ranging from 1.56 to 25 μg/ml. In general the activity of these synthetic analogues increased in direct proportion to the number of C-atoms in the cyclic ring. Among these compounds, 6-cyclopentylmethylsalicylic acid (**23**) was the least active analogue with an MIC of 25 μg/ml. The maximum activity for this series was found for 6-cyclododecylmethylsalicylic acid (**26**) which demonstrated an MIC of 1.56 μg/ml. The results clearly show that the activity of the synthetic analogue **26** against *S. mutans* is comparable with that of the standard naturally occurring anacardic acid ($C_{15:3}$).

Analogue **24** inhibited the growth of *S. mutans* at a concentration of 25 μg/ml, which was less active than 6-cyclohexylethylsalicylic acid (**27**) that has an additional C-atom in the linker chain between the phenyl and cyclohexyl rings in the C-6 side chain. The MIC of **27** was 3.13 μg/ml for *S. mutans.* A further most interesting observation was that 6-cyclooctylmethylsalicylic acid (**25**) with an MIC against *S. mutans* of 6.25 μg/ml, exhibited stronger activity than either that of cyclopentylmethyl (**23**) or cyclohexylmethylsalicylic acid (**24**) (see Table 2).

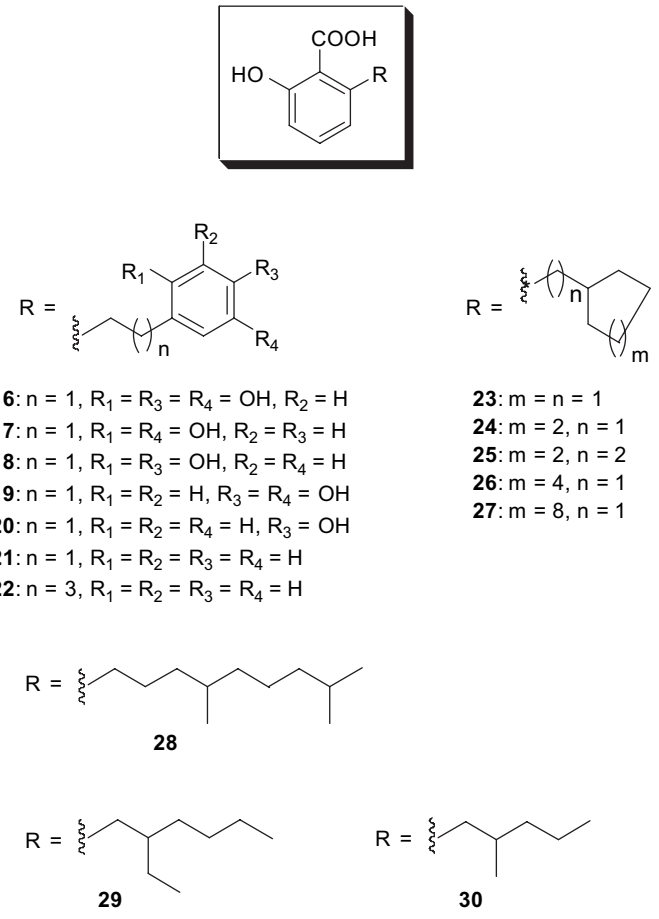


Fig. 2. Structures of anacardic acid derivatives.

The maximum activity of the C-6 branched alkanyl side chain anacardic acid analogues **28–31** against *S. mutans* was found with 6-(4′,8′-dimethylnonyl)salicylic acid (**28**) which had a MIC of 0.78 μg/ml. This rather good activity of **28** was thus double that of the best natural anacardic acid ($C_{15:3}$). With an MIC of 12.5 μg/ml, 6-(2′-methylhexyl)salicylic acid (**30**) was the least active of the branched side-chain anacardic acid analogues studied in this project. The MIC of analogue **29** having a C_9H_{19} branched side-chain was 3.13 μg/ml against *S. mutans* which is comparable with that of anacardic acid ($C_{12:0}$) [7]. It thus appears that the presence of a branched side-chain at C-6 in the salicylic acid nucleus plays a significant role in increasing the antibacterial activity of the 6-alkylsalicylic acids (Fig. 2).

3. Conclusions

On the basis of the data obtained, it may be concluded that antibacterial anacardic acids act primarily as surface-active agents to disrupt the native membrane-associated function of the integral proteins (physical disruption of the membrane) [11]. However, biochemical mechanisms may also be responsible for eliciting this activity and should therefore not be ruled out [14]. In addition, it appears that maximum activity

can be obtained when the most appropriate balance between hydrophilic and hydrophobic moieties is attained. Therefore, the alkyl side chain plays a direct role in the modulation of the activity. Currently, however, the precise explanation for the role of the alkyl side-chain remains unclear. It should further be borne in mind that the relevance of the in vitro experiments in simplified systems, when extended to include the complex interaction between anacardic acids and bacterial systems representing the real interactions, has to be carefully considered before any final pronouncements can be made.

Anacardic acid ($C_{15:0}$) (**3**) was previously reported to show high selectivity toward Fe^{2+} and Cu^{2+} [19]. This implies that metal ions might also play a significant role in antimicrobial activity by reducing their availability for bacteria [20]. It is considered that this possibility is rather unlikely since anacardic acid ($C_{15:0}$) (**3**) did not show any antibacterial activity against *S. mutans* even up to 800 μg/ml. However, due to the paucity of more investigative information, this hypothesis may not be totally excluded. In addition, metal chelation may play a significant role in determining the antioxidant activity [21]. The chelation ability, rendering the metal ions inactive to participate in free radical generating reactions, should be of considerable importance to advance their antioxidant activities. Thus, the salicylic acid moiety is also responsible for eliciting antioxidant properties without prooxidant effects [22–25]. In view of the increasing importance of controlling specific bacteria in the mouth, viz. *S. mutans*, studies on the branched side chain anacardic acid analogues may lead to new anticavity drugs with anti-*S. mutans* activity.

4. Experimental

4.1. Chemicals

Anacardic acids and their analogues were available in our laboratory from previous investigations [6–9,18], and synthesis of the 6-alkylsalicylic acid analog has been was previously described [12,13]. Nonyl 2-hydroxybenzoate (**7**) was synthesized by the method previously reported [26]. Salicylic acid and 1,1-diphenyl-2-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. Preparation of hexyl 2-hydroxybenzoate (*6*)

Hexyl 2-hydroxybenzoate was synthesized as follows [26]. A mixture of 2-benzyloxybenzoic acid (200 mg, 0.88 mmol) [27], 1-hexanol (108 mg, 1.06 mmol), and triphenylphosphine (350 mg, 1.33 mmol) in tetrahydrofuran (4 ml) was cooled to 0 °C and treated with diisopropyl azodicarboxylate (214 mg, 1.06 mmol). After being stirred for 2 h at room temperature, the solvent was removed in vacuo. The residue was subjected to silica gel chromatography and the product eluted with 1–8% EtOAc–hexane to give the corresponding ester as white solid, which was used in the next step without further purification. The ester was hydrogenated over 20% $Pd(OH)_2$ on carbon (10 mg) in 1% AcOH–EtOAc (4 ml) for 12 h. Filtration through Celite and concentration followed by silica gel chromatography with EtOAc–hexane (1–40%) as eluant gave pure hexyl 2-hydroxybenzoate (**6**) in 83% yield (2 steps) as a colorless oil (95 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.91 (t, $J = 6.8$ Hz, 3H), 1.34 (m, 4H), 1.45 (quin, $J = 6.8$ Hz, 2H), 1.79 (quin, $J = 6.8$ Hz, 2H), 4.35 (t, $J = 7.0$ Hz, 2H), 6.88 (ddd, $J = 2.0$, 7.0, 8.0 Hz, 1H), 6.98 (dd, $J = 2.0$, 9.0 Hz, 1H), 7.45 (ddd, $J = 2.0$, 7.0, 9.0 Hz, 1H), 7.84 (dd, $J = 2.0$, 8.0 Hz, 1H), 10.84 (s, 1H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.0, 22.5, 25.5, 28.5, 31.4, 65.5, 112.7, 117.6, 119.0, 129.4, 135.6, 161.7, 170.2 ppm; IR (CCl_4): 3185, 2850, 1665, 1470, 1290, 1210, 1140, 1070 cm^{-1}; EI-MS (*m*/*z*): 222 (M^+).

4.3. Microorganisms and media

S. mutans ATCC 25175 and *E. coli* ATCC 9637 were obtained from American Type Culture Collection (Manassas, VA). The freeze–dried culture of *S. mutans* was inoculated into 3.7% brain heart infusion (BHI) broth purchased from Difco Lab. (Detroit, MI) and incubated stationary for 2 days at 37 °C before the assay. Although only one strain of *S. mutans* was tested, compounds active against this strain are expected to retain a similar order of activity against a variety of strains of this species. In the case of *E. coli*, NYG broth, (0.8% nutrient broth, 0.5% yeast extract, and 0.1% glucose) was used. Nutrient broth was obtained from BBL Microbiology System (Cockeysville, MD) and yeast extract was purchased from Difco Lab.

4.4. Antibacterial assays

The assay was performed by a broth dilution method as previously reported [8]. Briefly, serial 2-fold dilutions of test compounds were made in DMF and 30 μl of each dilution was added to 3 ml of BHI broth. This test broth was then inoculated with 30 μl of a 2-day-old culture of *S. mutans*. The highest concentration used for the assay was 200 μg/ml, unless otherwise specified, because of solubility limitation in the water-based media of some of the samples. The lowest concentration of the test compound resulting in complete inhibition of visible growth after 2 days of incubation at 37 °C represented the MIC. 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 [16].

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