Antibacterial Activity of Long-Chain Alcohols against *Streptococcus mutans*

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The antibacterial activity against *Streptococcus mutans* of a number of naturally occurring compounds was tested. The emphasis was placed on a series of long-chain alcohols to gain new insight into their structural functions. Maximum activity seems to depend on the hydrophobic chain length from the hydrophilic hydroxyl group. Among the alcohols tested, 1-tridecanol was found to be the most effective for controlling this cariogenic bacterium.

INTRODUCTION

In our continuing search for antimicrobial agents from plants, a number of active principles have been isolated. Among the active compounds characterized against *Streptococcus mutans* are many alcohols. For example, linalool, nerolidol, geraniol, 1-octanol, and α-terpineol from green tea flavor (Nose et al., 1971) are among those whose antimicrobial activity has been reported (Kubo et al., 1992a). In addition, their esters, especially acetates, are common. For example, a large number of α-terpinyl and linalyl acetates were identified as antimicrobial substances in cardamom seed flavor (Kubo et al., 1991). In fact, free and esterified alcohols occur widely in nature, *e.g.*, in fruit (Bauer et al., 1990). The antimicrobial activity of alcohols has been previously reported, but studies have been limited to short-chain ($<C_6$) alcohols, almost exclusively ethanol (Ingram and Buttke, 1984). The antimicrobial activity of long-chain alcohols has been demonstrated only with restricted microorganisms because of their limited solubility in water, although the alcohols have higher activity compared to the corresponding acids and aldehydes (Kabara et al., 1972).

In previus papers, it has been explained that dental caries can be prevented by eliminating *S. mutans*, since many recent studies have concluded that *S. mutans* is the primary bacterium causing dental caries (Hamada and Slade, 1980; de Jong et al., 1984; Loesche, 1986). The difficulties associated with eliminating this cariogenic bacterium by means of chemicals are easily recognized (Fitzgerald, 1972). Among them, the safety of chemicals seems to be the most important, since *S. mutans* resides in the mouth. Hence, the antibacterial agents isolated from edible plants, food spices, and beverages seem to be superior compared to nonnatural products. A large number of antibacterial agents against *S. mutans* have been characterized in these sources (Kubo et al., 1991, 1993a). On the basis of the continuing accumulation of these kinds of data, we have become aware of an ambiguous rule in their structure–activity relationship: the activity of the compounds characterized against *S. mutans* seems to be due to a balance between the hydrophilic and hydrophobic portions of the molecule. To gain new insights into the role of the hydrophobic portions in the activity, a series of simple aliphatic alcohols was studied as a model. These alcohols seem to be an ideal group of molecules for this kind of study because of their structural simplicity and availability. The hydrophilic hydroxyl group is common in all of the molecules so that only the hydrophobic alkyl groups need to be investigated.

MATERIALS AND METHODS

Chemicals. Totarol (Kubo et al., 1992b; Ying and Kubo, 1992), polygodial, anethole (Kubo and Taniguchi, 1988; Kubo and Himejima, 1991, 1992), indole, δ-cadinene, β-caryophyllene, geraniol, nerolidol, linalool, α-terpineol, 1-octanol, caffeine (Kubo et al., 1992a), 1,8-cineol, α-terpinene, linalyl acetate, terpinyl acetate (Kubo et al., 1991), abietic acid, α-pinene, β-pinene, limonene, β-ionone, longiforene (Himejima et al., 1992), eugenol, methyleugenol, dodecanoic acid (Himejima and Kubo, 1992), octanoic acid, geranylacetone, α-ionone (Kubo et al., 1993a), and crinitol (Kubo et al., 1992c) were from previous works. All of the other substances employed for the assay were of commercial sources. For the assay, all of the substances were first dissolved in *N,N*-dimethylformamide (DMF) purchased from EM Science (Gibbstown, NJ).

Microorganism and Medium. The bacterium *S. mutans* ATCC 25175 used for the experiment was purchased from American Type Culture Collection (Rockville, MD). The freeze-dried culture of *S. mutans* was inoculated into 3.7% brain heart infusion broth (BHI, Difco) and incubated stationary for 2 days at 37 °C before the assay. Although only one strain of *S. mutans* was tested, a compound active against one strain is expected to retain a similar order of activity against a variety of strains of this species. On the other hand, the possibility of resistance among strains is always present. With these considerations in mind, the experiments were carried out.

Antibacterial Assay. The minimum inhibitory concentration (MIC) was determined by the broth dilution method as previously described (Kubo et al., 1992a–c). Briefly, BHI broths (3 mL) incorporating serial 2-fold dilutions of the test compound were inoculated with 30 μL of a 2-day-old culture of *S. mutans*. They were incubated at 37 °C for 2 days, and the MIC was recorded as the lowest concentration of test compund to inhibit growth. The highest concentration tested was 800 μg/mL, unless otherwise specified, because of the compounds' solubility limitation in the water-based media.

The time–kill curve method was used to study the bactericidal effects of compounds. A 30-μL aliquot of a 2-day-old culture of *S. mutans* was inoculated into 3 mL of BHI broth containing the appropriate concentrations of the test compound. The initial population was $1–8 \times 10^5$ colony forming units (CFU)/mL. The test cultures were incubated stationary at 37 °C for a 24-h period. At selected time intervals, samples from the test culture were taken, serially diluted in sterile saline, and plated onto BHI agar. The plates were incubated at 37 °C for 2 days before the colonies formed were counted.

Combination studies were performed by a broth dilution checkerboard method (Norden et al., 1979). A series of 2-fold dilutions of the compound was tested in combination with serial

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Table I. Antibacterial Activity of Phytochemicals against *S. mutans*

compound tested	MIC (μg/mL)	compound tested	MIC (μg/mL)
totarol (1)	0.78	limonen	100
caffeine	>400	α-pinene	50
indole	800	β-pinene	200
geraniol (4)	400	α-terpinene	100
nerol (5)	400	α-humulene	>800
myrcenol (6)	400	δ-cadinene	800
citronellol (7)	400	β-caryophyllene	>1600
ipsdienol	>800	longiforene	100
linalool (10)	1600	*cis*-jasmone	800
menthol (18)	400	α-ionone	100
α-terpineol (17)	400	β-ionone	100
borneol	800	1,8-cineol	>800
farnesol (2)	12.5	α-terpinyl acetate	200
nerolidol (3)	25	linalyl acetate	400
cadinol (16)	12.5	7-hydroxycadalene (19)	12.5
crinitol (9)	50	thymol (20)	200
geranylacetol (13)	25	eugenol	400
geranylacetone (11)	50	methyleugenol	400
farnesylacetol (14)	>800	anethol	200
farnesylacetone (12)	>800	abietic acid	50
geranylgeraniol (8)	>800	3-tridecanone	>800
lupeol	>800	menthone	800
β-amyrin	>800	citral	>800
cholesterol	>800	citronellal	800
β-sitosterol	>800	dodecanal	>800
stearic acid	>800	dodecanoic acid	>800
polygodial	100	octanoic acid	1600

2-fold dilutions of the other compound. The MIC was determined using the same method as described above.

All experiments were carried out at least twice.

RESULTS AND DISCUSSION

The antibacterial activities against *S. mutans* of various phytochemicals are listed in Table I. Some data were taken from our previous papers (Himejima and Kubo, 1992; Kubo et al., 1991, 1992a–c, 1993a). The most potent group of compounds among those characterized were anacardic acids isolated from the cashew *Anacardium occidentale* (Anacardiaceae) apple juice (Himejima and Kubo, 1991). We have previously described their usefulness as anticaries agents based on our extensive studies (Muroi and Kubo, 1993). Although the activity is potent, their structures are rather too complicated for the structural function study (Kubo et al., 1993b). Totarol (1), isolated from the bark of an ornamental tree *Podocarpus nagi* (Podocarpaceae), showed even more potent activity against this cariogenic bacterium. However, this diterpenoid was not further studied because of its limited availability and, more importantly, *P. nagi* is not edible in any form. In this paper we discuss it only for comparison purposes. All of the other compounds characterized did not warrant further study because of one or more of the following problems: lack of potency, structural complexity, or limited availability. On the basis of these considerations, the aliphatic alcohols seem to be superior not only because of their structural simplicity but also because of their availability.

The study was conducted primarily with a series of C_6–C_{20} aliphatic chain alcohols. The activities against *S. mutans* of the alcohols of the C_6–C_{20} chain lengths are listed in Table II. The C_7–C_{13} chain lengths exhibited activity against *S. mutans* as illustrated in Figure 1. The MICs ranged from 6.25 to 800 μg/mL. Optimum activity was found in 1-dodecanol (C_{12}) and 1-tridecanol (C_{13}), with the MIC of both being 6.25 μg/mL. Interestingly, this activity dropped off much more rapidly above C_{13} than below. Thus, 1-tetradecanol (C_{14}) no longer showed any activity against *S. mutans* up to 800 μg/mL. There is an apparent correlation between the antibacterial activity against this cariogenic bacterium and the carbon chain lengths from the hydrophilic hydroxyl group for the maximum activity against *S. mutans* should be less than C_{14} but as close to C_{14} as possible.

Table II. Antibacterial and Bactericidal Activity of Alcohols against *S. mutans*

alcohol tested	MIC[a]	MBC[a]	alcohol tested	MIC[a]
1-hexanol	>800	–[b]	2-hexanol	800
1-heptanol	800	>800	2-heptanol	>800
1-octanol	400	800	2-octanol	800
1-nonanol	100	400	2-nonanol	800
1-decanol	50	100	2-decanol	100
1-undecanol	25	100	2-undecanol	12.5
1-dodecanol	6.25	12.5	2-tetradecanol	>800
1-tridecanol	6.25	6.25	2-hexadecanol	>800
1-tetradecanol	>800	–		
1-pentadecanol	>800	–	3-hexanol	>800
1-hexadecanol	>800	–	3-heptanol	>800
1-pentadecanol	>800	–	3-octanol	>800
1-octadecanol	>800	–	3-nonanol	400
1-eicosanol	>800	–	3-tridecanol (15)	12.5

[a] μg/mL. [b] –, not tested.

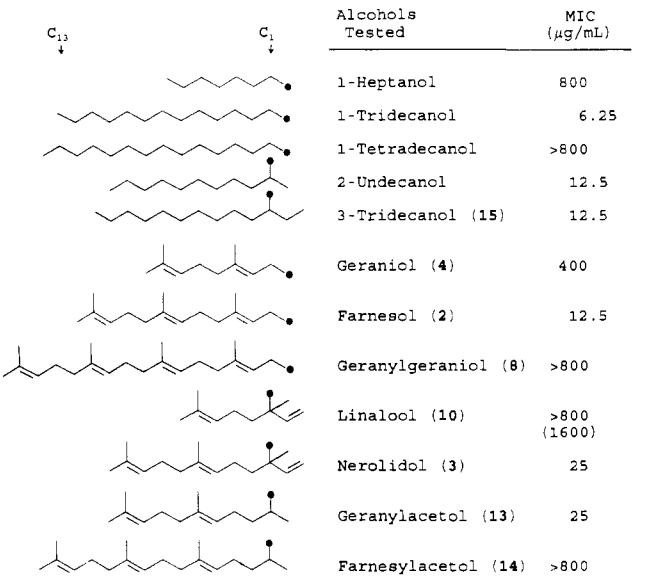
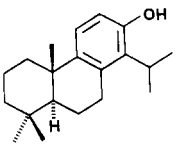


Figure 1. Structure–antibacterial activity relationships of long-chain alcohols against *S. mutans*: (●) hydrophilic hydroxyl group.

The results seem to be generally applicable to naturally occurring isoprene long-chain alcohols. For example, a sesquiterpene alcohol, farnesol (2), was easily selected as an active compound against *S. mutans* since its chain length was comprised of 12 carbon atoms and was found to exhibit the maximum activity against this cariogenic bacterium, while a similar sesquiterpene alcohol, nerolidol (3), exhibited less activity since its chain length from the hydroxyl group consisted of 10 carbon atoms. On the other hand, a monoterpene alcohol, geraniol (4), of which the chain length comprised 8 carbon atoms, showed less activity than the former two sesquiterpene alcohols (Figure 1). In addition, its congeners, nerol (5), myrcenol (6), and citronellol (7), all exhibited the same MIC since all have the same chain length comprised of 8 carbon atoms. Hence, their activities are comparable to that of 1-octanol. The MICs of these isoprene long-chain alcohols are comparative to the corresponding straight-chain alcohols. Geranylgeraniol (8) did not exhibit any activity against *S. mutans* up to 800 μg/mL, while crinitol (9), which possesses an additional hydroxyl group at C-9, showed activity with a MIC of 50 μg/mL (Kubo et al., 1992c).



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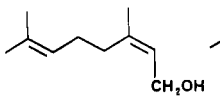

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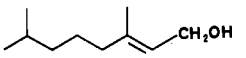

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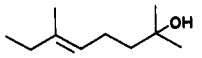

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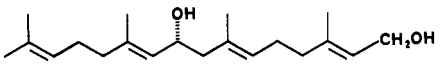

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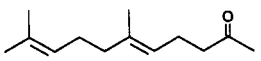

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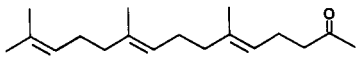

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The MICs, which were obtained by measuring the turbidity after 48 h of incubation, do not fully characterize the activity of these alcohols. Needless to say, it would be superior if the activity is bactericidal rather than bacteriostatic. In a previous paper, the MIC of linalool (**10**) was also established as the minimum bactericidal concentration (MBC) by the time–kill curve method (Kubo et al., 1993). Therefore, we studied the activity of 1-dodecanol and 1-tridecanol against the cariogenic bacterium in more detail. As illustrated in Figure 2, cultures of *S. mutans*, with a cell density of 3×10^5 CFU/mL, were exposed to different concentrations of the alcohols. The most notable result was that their MBCs were found to differ 2-fold. Thus, as far as the MICs are concerned, both alcohols showed the same concentration of 6.25 μg/mL; however, the MBC of 1-tridecanol was found to be 6.25 μg/mL, while that of 1-dodecanol was 12.5 μg/mL. It is now apparent that 1-tridecanol is more effective for controlling *S. mutans* than 1-dodecanol. Thus, optimum activity was exhibited when the chain length from the hydrophilic hydroxyl group comprised 13 carbon atoms.

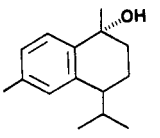
The MBCs were also obtained as follows: after the MIC was determined, a 30-μL aliquot was taken from the test tube which showed no turbidity and added into 3 mL of the alcohol-free fresh medium. After 48 h of incubation, the MBC was determined as the lowest concentration of the alcohol in which no recovery of *S. mutans* was observed. The results were compatible with those obtained by the time–kill curve method as shown in Table II.

The two most common naturally occurring acetates, linalyl acetate and α-terpinyl acetate, were also found to have activity against *S. mutans* with MICs of 400 and 200 μg/mL, respectively. Interestingly, these MICs are more potent than those of the corresponding alcohols (Kubo et al., 1991). For example, the MIC of linalyl acetate is 4-fold more than that of linalool (**10**). We expected to find similar results with 1-dodecanol and 1-tridecanol, which showed the most potent activity against *S. mutans*. Hence, dodecanyl acetate and tridecanyl acetate were also tested. Unexpectedly, neither acetate showed any activity up to 800 μg/mL.

In general, the alcohols have higher activity compared to the corresponding acids or aldehydes (Kabara et al., 1972). For example, 1-octanol was found to be more efficient than octanoic acid or octanal. On the basis of this kind of information, the ketone group of geranylacetone (**11**) and farnesylacetone (**12**) was reduced to geranylacetol (**13**) and farnesylacetol (**14**), respectively. As expected, geranylacetol exhibited activity against this cariogenic bacterium with a MIC of 25 μg/mL, while farnesylacetol did not show any activity up to 800 μg/mL (Figure 1). Apparently, the carbon chain length of 14 carbon atoms from the hydrophilic hydroxyl group of the latter alcohol exceeded the chain length of 13 carbon atoms for the MIC. Similarly, 3-tridecanol (**15**) exhibited activity against this cariogenic bacterium with a MIC of 12.5 μg/mL, while 3-tridecanone did not show any activity up to 800 μg/mL.

As mentioned above, the activity dropped off suddenly above the chain length for the maximum activity. Thus, 1-tridecanol (C_{13}) was found to be the most effective against *S. mutans* with the MIC of 6.25 μg/mL, while 1-tetradecanol (C_{14}) did not show any activity even up to 800 μg/mL.

In addition, the position, number, and stereochemistry of double bonds seem to affect the activity in some ways. Obviously, more work is needed to clarify these. The results obtained so far are somehow similar to long-chain fatty acids (Kabara, 1979). In contrast to the fatty acids, alcohols have many more diverse structures. For example, cyclic alcohols occur commonly in nature. The volume of the hydrophobic portions also seems to affect the activity. For example, a bicyclic sesquiterpene alcohol, cadinol (**16**),



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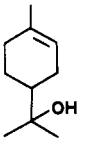

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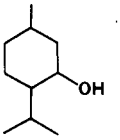

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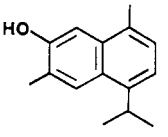

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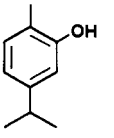

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showed more potent activity than the monocyclic monoterpene alcohols α-terpineol (**17**) and menthol (**18**). Similarly, a bicyclic sesquiterpene, 7-hydroxycadalene (**19**), exhibited stronger activity than the monocyclic thymol (**20**), while a tricyclic diterpenoid, totarol (**1**), showed even more potent activity. However, two common steroids, cholesterol and β-sitosterol, and terpenoids, lupeol and β-amyrin, did not show any activity up to 800 μg/mL. Interestingly, several hydrocarbons such as limonene, α-pinene, β-pinene, longiforene, δ-cadinene, and β-caryophyllene also showed some activity. The volume of hydrophobic portions seems to affect the activity in some way. However, the role of the molecules' volume in the activity remains to be studied.

Lastly but most importantly, 1-tridecanol and 1-dodecanol were combined with several other substances to enhance the total activity. Combining more than two

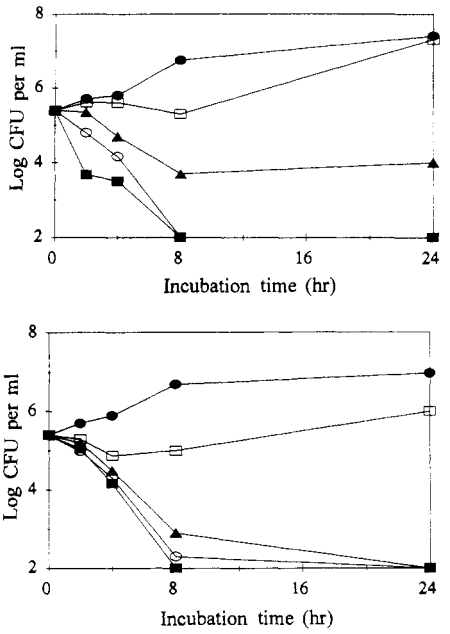

Figure 2. Bactericidal effects of 1-dodecanol (upper graph) and 1-tridecanol (lower graph) on *S. mutans*. A 48-h culture was inoculated into the BHI broth containing 0 (●), 3.13 (□), 6.25 (▲), 12.5 (○), and 25 μg/mL (■) of the two alcohols.

substances seems to be a more efficient approach in using renewable natural products, although the rationale for selecting the other substances being combined is still in a preliminary stage. The purpose of the combination is not only to enhance the activity specifically against *S. mutans* but also, more importantly, to hinder the development of resistance mechanisms in *S. mutans*. The selection of the other substances was based largely on our previous studies (Kubo et al., 1992a,b; Kubo and Himejima, 1991). For example, the antibacterial activity of δ-cadinene against *S. mutans* was significantly enhanced when it was combined with a sublethal amount of indole. Hence, the half-MIC of anethole, indole, or anacardic acid was combined with 1-tridecanol or 1-dodecanol. As a result, none of them showed dramatic synergistic effects to these alcohols. Thus, all of the combinations were only additive. However, the MIC of an acyclic diterpene alcohol, crinitol (**9**), was increased 64-fold in combination with the half-MIC of BHT against *S. mutans* (Kubo et al., 1992c).

An aim of the current experiments is to find antibacterial agents from edible plants, food spices, and beverages for oral care products. Both 1-nonanol (C_9) and 1-octanol (C_8) are reported in various oranges, teas, and cheeses, and 1-dodecanol (C_{12}) is found in the oil of Mexican limes. In addition, these alcohols have also been used as flavor components in common foods such as chewing gum, candy, and baked goods (Furia and Bellanca, 1975) so that their use, especially that of 1-dodecanol which showed maximum antibacterial activity against *S. mutans*, for oral care products as anticaries agents should be admissible.

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