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NATURALLY OCCURRING ANTIACNE AGENTS

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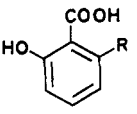
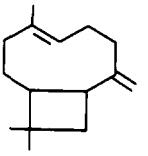
ABSTRACT.—Antibacterial activity of various secondary metabolites from plants against *Propionibacterium acnes* was tested. In addition, the study of combinations of compounds to enhance the total activity against this follicular bacterium was investigated. A series of long-chain alcohols was studied in great detail to gain new insights into the role of the hydrophobic alkyl groups in the activity.

Acne is a follicular disorder of the skin occurring in specialized pilosebaceous units on the face and neck. An abnormality of the keratinizing epithelium of these follicles, thought to be due to the action of sebum synthesized and secreted by the androgen-sensitive sebaceous glands, leads to inflammation induced by the follicular bacterium *Propionibacterium acnes*. Therapy, involving treatments that modify these pathogenic factors, includes drugs with antibacterial, antikeratinizing, and antiseborrheic actions (1–3). *Pr. acnes* is generally considered to be non-pathogenic to man, although it has been identified in pure and mixed cultures as a cause of serious infections, especially in conjunction with foreign body implants (4). In our continuing search for antimicrobial agents from plants, *Pr. acnes* is often the most susceptible among the microorganisms tested. Hence, a large number of active principles have been isolated from various plants (5–7). For example, anacardic acids **1**–**4** isolated from the cashew *Anacardium occidentale* (Anacardiaceae), apple, nut, and nut shell oil (6); β-caryophyllene [**5**] and δ-cadinene [**6**] identified in green tea flavor (7); and totarol [**7**] isolated from the bark of *Podocarpus nagi* (Podocarpaceae) (8), showed potent activity against *Pr. acnes*. Their minimum inhibitory concentrations (MICs) ranged from 0.39 to 6.25 μg/ml. As far as the MICs are concerned, they may be potent enough to be considered for practical use. However, in most cases, the activity of natural products usually lacks potency. Nevertheless, they are all biodegradable and, more importantly, renewable. The application of such renewable natural substances to cosmetic products has produced a keen interest, and studies to enhance the activity are needed. Combining more than two compounds in order to enhance the total activity seems to be a most promising strategic approach to this problem (9–11). Most importantly, this approach may hinder the development of resistant mechanisms in *Pr. acnes*. In order to proceed, understanding the effects of the antibacterial compounds on bacterial systems and the biochemical changes involved is essential. This led us to initiate a systematic structure-antibacterial activity relationship study against this follicular bacterium with a series of simple model compounds.



R=

1 $C_{15:3}$, 6-[8(*Z*),11(*Z*),14-Pentadecatrienyl]salicylic acid
2 $C_{15:2}$, 6-[8(*Z*),11(*Z*)-Pentadecadienyl]salicylic acid
3 $C_{15:1}$, 6-[8(*Z*)-Pentadecenyl]salicylic acid
4 $C_{15:0}$, 6-Pentadecylsalicylic acid



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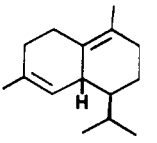

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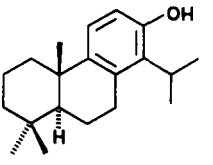

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RESULTS AND DISCUSSION

Antibacterial activity against *Pr. acnes* of various natural products is listed in Table 1. Some of the data were taken from our previous reports (5,7,8,10,12,13). Among the active compounds isolated, totarol [**7**] showed the most potent activity, with an MIC of 0.39 μg/ml, followed by anacardic acids **1–4** with MICs of 0.78 μg/ml (Table 2). Although totarol was found to be the most effective against *Pr. acnes,* it could not be used for further structure-activity relationship study because of its limited availability. As two sesquiterpene hydrocarbons, δ-cadinene [**6**] and β-caryophyllene [**5**], also showed potent activity against *Pr. acnes*, various naturally occurring monoterpene and sesquiterpene hydrocarbons were tested. The two additional sesquiterpene hydrocarbons tested, α-humulene and longiforene, exhibited potent activity with MICs of 3.13 and

TABLE 1. Antibacterial Activity of Various Phytochemicals Against *Propionibacterium acnes* (ATCC 11827).

Compound tested	MIC[a]	Reference	Compound tested	MIC[a]	Reference
Polygodial	25		Geraniol	400	7
Totarol [**7**]	0.39	8	Borneol	200	
Hinokitiol	12.5		Menthol	400	
Abietic acid	25		1-Octanol	200	7
Nagilactone E	>800	13	α-Terpineol	100	7
Nagilactone C	>800	13	4-Terpineol	800	
α-Nagilactone F	>800	13	β-Sitosterol	>800	
Caffeine	>400	7			
Indole	200	7	Cholesterol	>800	
Anethole	100	10	δ-Cadinene [**6**]	3.13	7
Eugenol	50	5	α-Humulene	3.13	
Methyleugenol	200	5	Longiforene	6.25	
Safrole	50	5	β-Caryophyllene [**5**]	6.25	7
Isosafrole	200		Limonene	50	5
α-Ansrone	100		Terpinolene	50	
Thymol	200		α-Pinene	25	
Linalool	200	7	β-Pinene	100	
Crinitol	25	12	3-Carene	50	
Nerolidol	25	7	(+)-Camphene	25	
Farnesol [**8**]	6.25		(−)-Camphene	25	
Geranylacetol	3.13		Camphor	200	
Farnesylacetol [**9**]	1.56		Farnesylacetone	>800	

[a]μg/ml.

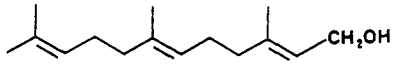
TABLE 2. Antibacterial Activity of Anacardic Acids and Their Analogues Against *Propionibacterium acnes* (ATCC 11827).

Compound tested	MIC (μg/ml)
6-[8(Z),11(Z),14-Pentadecatrienyl]salicylic acid [**1**]	0.78
6-[8(Z),11(Z)-Pentadecadienyl]salicylic acid [**2**]	0.78
6-[8(Z)-Pentadecenyl]salicylic acid [**3**]	0.78
6-Pentadecylsalicylic acid [**4**]	0.78
Salicylic acid	400
6-Methylsalicylic acid	200
6-Pentylsalicylic acid	100
6-Octylsalicylic acid	3.13
6-Decylsalicylic acid	1.56
6-Dodecylsalicylic acid	0.39
6-Eicosylsalicylic acid	>800

6.25 μg/ml, although monoterpene hydrocarbons, limonene, terpinolene, α-pinene, β-pinene, 3-carene, camphor, and camphenes were found to show at most moderate activity, with MICs ranging from 25 to 200 μg/ml. Their potency did not warrant further study.

In the case of anacardic acids, interestingly, the degree of unsaturation in the side chain of the anacardic acids did not affect the activity against *Pr. acnes*. Thus, the four natural anacardic acids **1**–**4** inhibited the growth of *Pr. acnes* at the same concentration, 0.78 μg/ml (14), as listed in Table 2. In contrast to this follicular bacterium, against all the other bacteria tested such as *Staphylococcus aureus, Streptococcus mutans, Bacillus subtilis,* and *Brevibacterium ammoniagenes*, an increase in the number of double bonds in the side chain increased the activity (14,15). A similar observation with long-chain fatty acids was reported (16). In addition, we have previously found that the length of the alkyl side chain of these anacardic acids **1**–**4** plays an important role in increasing the antibacterial activity (14). The maximum activity against *Pr. acnes* was found to occur at the C_{12} side chain, with an MIC of 0.39 μg/ml (Table 2). Thus, the activity is dependent on the side chain length. All the other aromatic compounds tested, such as anethole, eugenol, thymol, α-asarone, methyleugenol, safrole, and isosafrole, which do not have a long side chain in the molecules, exhibited only weak activity against this follicular bacterium. Their MICs ranged between 50 and 200 μg/ml (Table 1). These results indicated that antimicrobial activity is largely affected by the side chain length. The differences in the chemical properties of these compounds of different side chain lengths are undoubtedly responsible. The mechanisms of the activity seem to be due to a balance between the hydrophilic and hydrophobic parts of the molecule.

Bioassay-guided fractionations have led to the isolation of a number of alcohols as antibacterial agents against *Pr. acnes*. In fact, free and esterified alcohols are known to occur widely in nature, e.g., in fruit (17). Although some naturally occurring alcohols, farnesol [**8**] and farnesylacetol [**9**], exhibited potent activity against *Pr. acnes*, most of



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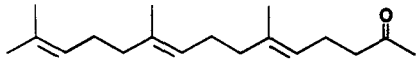

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those, linalool, nerolidol, geraniol, menthol, borneol, 4-terpineol, 1-octanol, α-terpineol, and crinitol (5,7,12), exhibited at most moderate activity (Table 1). Their potency may not warrant further study. However, alcohols are among the most versatile of all organic compounds. Their structural diversity is ideal (aliphatic alcohols especially seem to be superior) for a model study in understanding their modes of action on a molecular basis because of their structural simplicity.

The study has been conducted primarily with a series of C_6 to C_{20} long-chain alcohols. The emphasis has been placed on saturated, primary alcohols because of their structural simplicity and availability of the samples. In addition, alcohols are known to have higher activity compared to the corresponding acids and aldehydes (18), but owing to their limited solubility in H_2O, the effectiveness of long-chain alcohols has been demonstrated only on limited microorganisms. In fact, the activity of long-chain alcohols against *Pr. acnes* has not yet been reported.

The initial aim was to analyze the susceptibility patterns of *Pr. acnes* to the 14 primary alcohols. The results are listed in Table 3. These alcohols are the most effective against Gram-positive bacteria, and possess little or no activity against Gram-negative bacteria. The alcohols of the C_7 to C_{16} chain lengths exhibited activity against *Pr. acnes*, with their MICs ranging between 0.78 and 800 μg/ml. The maximum activity occurred at 1-pentadecanol (C_{15}) and 1-hexadecanol (C_{16}), with the MIC of both being 0.78 μg/ml. Interestingly, the activity suddenly dropped off above C_{16}. In other words, 1-heptadecanol (C_{17}) no longer showed any activity up to 800 μg/ml. It is evident from these results that antibacterial activity against *Pr. acnes* of free long-chain alcohols depends on the carbon chain lengths. The differences in the chemical properties of these alcohols are undoubtedly responsible. The extent of their antibacterial activity was associated with the relative contributions of the hydrophilic and hydrophobic components of the molecule (19). In addition, because many naturally occurring alcohols are secondary alcohols, some available secondary alcohols were also tested. The results (Table 3) were similar to those of primary alcohols. Among them, 2-hexadecanol showed the most activity with an MIC of 0.78 μg/ml.

As mentioned above, *Pr. acnes* is susceptible to the long-chain alcohols whose MICs ranged between 0.78 and 800 μg/ml. Among the alcohols tested, 1-pentadecanol, 1-hexadecanol, and 2-hexadecanol may be potent enough to be considered for practical use

TABLE 3. Antibacterial and Bactericidal Activity (MIC and MBC, μg/ml) of Alcohols Against *Propionibacterium acnes* (ATCC 11827).

Alcohol tested	MIC	MBC	Alcohol tested	MIC
1-Hexanol	>800	—[a]	2-Hexanol	800
1-Heptanol	400	800	2-Heptanol	800
1-Octanol	200	400	2-Octanol	800
1-Nonanol	100	200	2-Nonanol	400
1-Decanol	25	50	2-Decanol	100
1-Undecanol	12.5	25	2-Dodecanol	12.5
1-Dodecanol	3.13	6.25	2-Tetradecanol	3.13
1-Tridecanol	1.56	3.13	2-Hexadecanol	0.78
1-Tetradecanol	1.56	1.56	2-Octadecanol	>800
1-Pentadecanol	0.78	1.56		
1-Hexadecanol	0.78	1.56	3-Hexanol	>800
1-Heptadecanol	>800	—[a]	3-Heptanol	800
1-Octadecanol	>800	—[a]	3-Octanol	400
1-Eicosanol	>800	—[a]	3-Nonanol	200

[a]Not tested.

as antiacne agents. It has been previously reported that free fatty acids and alcohols of the C_8 to C_{14} chain lengths have caused skin irritant effects (20), while these effects have not been observed with alcohols that have chain lengths greater than C_{14}. Therefore, our emphasis was placed on 1-pentadecanol (C_{15}), 1-hexadecanol (C_{16}), and 2-hexadecanol (C_{15}), which all have carbon chain lengths greater than C_{14} from the hydrophilic hydroxyl group but shorter than C_{17}. They all exhibited potent activity against *Pr. acnes* with MICs being 0.78 μg/ml. Interestingly, these alcohols showed activity against only *Pr. acnes*, but not the other microorganisms when tested up to 800 μg/ml. Thus, the growth of *Pr. acnes* was inhibited by alcohols of slightly longer chains than those inhibiting the growth of the other microorganisms. This may allow us to better differentiate toxic effects against this specific target bacterium, *Pr. acnes*. In skin surface lipid film, some microorganisms, called normal skin microflora, reside in harmony. *Pr. acnes*, which is one of the normal skin microflora, seems to play an important role in maintaining this ecosystem. We need to eliminate this follicular bacterium to cure acne without destroying this ecosystem. 1-Pentadecanol, 1-hexadecanol, and 2-hexadecanol may be used as selective antiacne agents.

The MICs, which were obtained by measuring the turbidity after 48 h of incubation, do not fully characterize the activity of these alcohols. Therefore, we studied in more detail the activity using 1-pentadecanol. Needless to say, it would be superior if the activity was bactericidal rather than bacteriostatic. Therefore, the effect of 1-pentadecanol was studied by the time-kill curve method. As illustrated in Figure 1, cultures of *Pr. acnes*, with a cell density of 1.4×10^6 colony forming units (CFU) per ml, were exposed to different concentrations of 1-pentadecanol. This alcohol showed bactericidal activity against *Pr. acnes* at 1.56 μg/ml. The effect of 1-pentadecanol was further studied in the lag and exponential growth phases. In Figure 2, it can be seen that 25 μg/ml of 1-pentadecanol showed bactericidal effect at both the lag and exponential growth phases, producing a >2 log units reduction in CFU per ml at 48 h of incubation after adding the alcohol. This concentration of 1-pentadecanol killed the *Pr. acnes* cells even at the exponential growth stage in which metabolic and enzymatic activities are high. However, this alcohol was not bactericidal at 6.25 μg/ml and was no longer active at 1.56 μg/ml on exponentially growing *Pr. acnes*.

The minimum bactericidal concentrations (MBCs) of the primary long chain alcohols were also established as follows: after determining the MIC, a 30 μl aliquot was

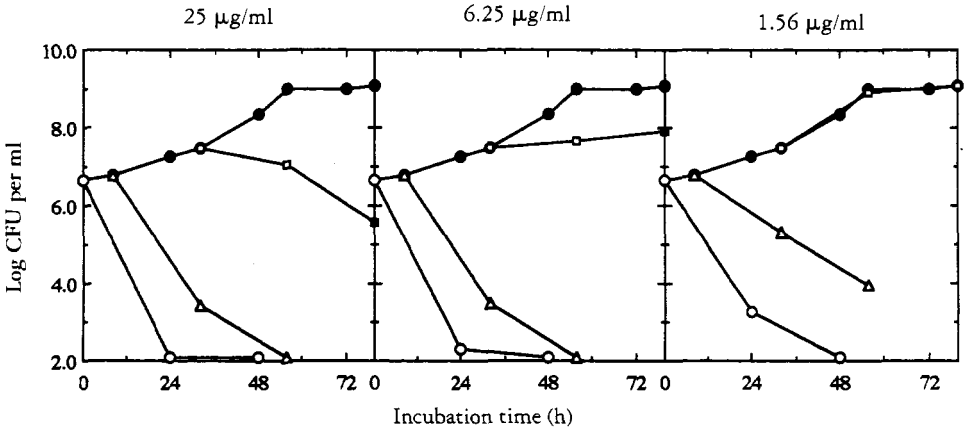

FIGURE 1. Effects of 1-pentadecanol on the growth of *Propionibacterium acnes* ATCC 11827. The number of viable cells was determined in NYG broth containing 0 (●), 0.39 (×), 0.78 (□), 1.56 (△), and 12.5 (○) μg/ml of 1-pentadecanol.

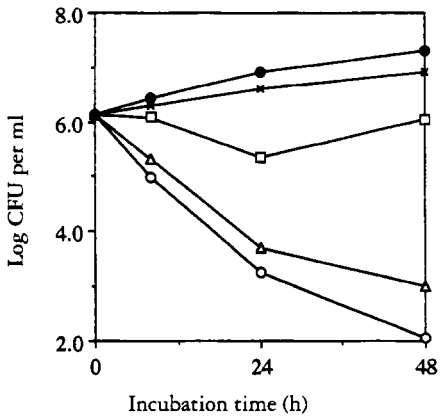

FIGURE 2. Effects of 1-pentadecanol on *Propionibacterium acnes* ATCC 11827 in the lag and exponential growth phase. 1-Pentadecanol was added at 0 h (○), 4 h (△), and 32 h (□) after the beginning of incubation. Control (●).

taken from each test tube that showed no turbidity and added to 3 ml of the alcohol-free fresh medium. After 2 days of incubation, the MBC was determined as the lowest concentration of the alcohol in which no recovery of *Pr. acnes* was observed. The results are listed in Table 3. The antibacterial activity of the primary alcohols was found to be bactericidal; the MBC to MIC ratios being no greater than two. The results are in general agreement with the above data obtained by the time-kill curve method.

From all of these findings we may postulate that the fluidity of the cell membrane can be disturbed maximally by the hydrophilic hydroxyl group of the long-chain alcohols. It could attach itself into the molecular structure of the membrane with the polar hydroxyl group oriented into the aqueous phase by hydrogen bonding and nonpolar carbon chain aligned into the lipid phase by dispersion forces. If so, interestingly, why does the additional CH_2 make a big difference in their activity? The potent activity of 1-hexadecanol (C_{16}) against *Pr. acnes* with an MIC of 0.78 μg/ml was no longer observed for 1-heptadecanol (C_{17}) up to 800 μg/ml. Nevertheless, their modes of action, particularly concerning interaction with membranes, still remain to be learned.

The activity of an antiacne agent is related to its use on lipid-rich regions of the skin. In a previous report, the effect of benzoyl peroxide against *Pr. acnes* was enhanced when it was tested in a high-lipid environment because of its superior solubility in a lipid (21). Since almost all the substances described here are highly lipophilic, a high-lipid environment might enhance their effects against this follicular bacterium.

Furthermore, *Pr. acnes* is known to produce a lipase which hydrolyzes skin surface lipids, most likely triglycerides, to free fatty acids (22,23). Interestingly, C_6–C_{16} free fatty acids inhibit the growth of *Pr. acnes*. Among them, dodecanoic acid (C_{12}) was reported to be the most effective (24) with an MIC of 50 μg/ml. Our recent experiment also found dodecanoic acid to be one of the most potent among the fatty acids tested (25). Unless the lipase does not require a specific substrate (lipid), esters may become potential antiacne agents. With this in mind, pentadecanyldodecanoate was synthesized to use as a solvent for the lipophilic antiacne agents described here, such as totarol, 1-pentadecanol, and 6-dodecanylsalicylic acid. This ester consists of 1-pentadecanol and dodecanoic acid,

which are among the most potent alcohols and acids, respectively, against *Pr. acnes*. The idea is that *Pr. acnes* lipase may hydrolyze it to two potent antiacne agents which, together with the added antiacne agents, kill the bacterium itself. As expected, the ester did not exhibit any activity against *Pr. acnes* up to 200 μg/ml by the broth dilution method. The highest concentration tested was 200 μg/ml because of its limited solubility in the H_2O-based medium. Although the lipase from *Pr. acnes* was not induced under this condition, available lipase from two fungi, *Rhizopus arrhizus* and *Candida cylindracea*, was tested. The growth of *Pr. acnes* was not affected by these lipases alone. In combination with 1000 units of the lipase, the ester itself became active, with an MIC of 1.56 μg/ml.

Lastly, but more importantly, some of the antiacne agents described here were combined with other substances in order to enhance their activity specifically against *Pr. acnes*. The combining of two or more compounds could not only enhance the total activity but also, more likely, hinder the development of resistance mechanisms of *Pr. acnes*. In a previous paper, we reported the combination effects between indole and δ-cadinene, and indole and β-caryophyllene against this bacterium, although the combinations were only additive (7). 1-Pentadecanol and 1-hexadecanol were also examined in combination with indole as well as anethole. However, the combinations of these alcohols were only additive. In the current study, anethole was found to enhance the activity of anacardic acid [**1**] and farnesol [**8**], as shown in Table 4. Thus, in combination with 100 μg/ml (=½ MIC) of anethole, the activity of anacardic acid and farnesol was increased 8- and 16-fold, lowering the MICs from 0.78 to 0.098 and 6.25 to 0.39 μg/ml, respectively. However, owing to the strong odor of anethole, this combination may not be completely agreeable. With this in mind, anacardic acid [**1**] was combined with ½ MIC of each β-caryophyllene [**5**] and farnesol [**8**], since both have potent activity against *Pr. acnes*, with both of their MICs being 6.25 μg/ml. As a result, β-caryophyllene and farnesol enhanced the activity of anacardic acid 4-fold. Interestingly, this synergism was vice versa. Thus, anacardic acid also increased the activity of β-caryophyllene and farnesol 4- and 8-fold,

TABLE 4. MICs of Anacardic Acid [**1**], Anethole, Farnesol [**8**], and β-Caryophyllene [**5**] in Combinations Against *Propionibacterium acnes* (ATCC 11827).

Compound[a]	MIC (μg/ml)			
	Anacardic Acid	Anethole	Farnesol	β-Caryophyllene
None	0.78	200	6.25	6.25
Anacardic Acid		25	0.78	1.56
Anethole	0.098		0.39	—[b]
Farnesol	0.2	50		—[b]
β-Caryophyllene	0.2	—[b]	—[b]	

[a]At ½ MIC.
[b]Not tested.

as shown in Table 4. For example, the MIC of anacardic acid was reduced from 0.78 to 0.2 μg/ml in combination with 3.13 μg/ml (=½ MIC) of farnesol. Similarly, the MIC of farnesol was lowered from 6.25 to 0.78 μg/ml when it was combined with 0.39 μg/ml (=½ MIC) of anacardic acid. An acyclic diterpenoid, crinitol, was isolated from a marine algae as an antibacterial principle; however, the lack of potency has limited the application of this compound. Hence, crinitol was combined with 0.39 μg/ml of anacardic acid. As a result, its MIC was reduced from 25 to 3.13 μg/ml.

EXPERIMENTAL

CHEMICALS.—Anacardic acids **1–4** (26) were previously isolated in our laboratory, and the serial analogues of anacardic acid were previously synthesized (14). Totarol [7] (8,27), crinitol (12), indole, δ-cadinene, β-caryophyllene, geraniol, nerolidol, linalool, α-terpineol, 1-octanol (7), polygodial, anethole (10), abietic acid, α-pinene, β-pinene, 3-carene, limonene, terpinolene, longiforene (28), safrole, eugenol, methyleugenol (13,25,26), nagilactones, α-asarone, isoeugenol (13), and caffeine (29) were from previous work. All of the other compounds employed were of commercial source. For the antibacterial experiments, all compounds were first dissolved in DMF obtained from EM Science (Gibbstown, NJ). Lipases were purchased from Sigma Chemical Co. (St. Louis, MO).

BACTERIUM AND MEDIUM.—The bacterium *Pr. acnes* ATCC 11827 used for the experiment was purchased from American Type Culture Collection (Rockville, MD). The NYG broth was used for the antibacterial assay and consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco), and 0.1% glucose. Although only one strain of *Pr. acnes* 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 MICs were determined by a broth dilution method as previously described (7,12). Briefly, serial twofold dilutions of test compounds were made in DMF, and 30 μl of each solution was added to 3 ml of NYG broth. This test broth was then inoculated with 30 μl of a two-day-old culture of *Pr. acnes*. Because of solubility limitations of the samples in DMF and/or the H_2O based media, the highest concentration used for the assay was 800 μg/ml unless otherwise specified. After 2 days of incubation at 37°, the growth of *Pr. acnes* was examined as a function of turbidity (OD at 660 nm). The lowest concentration of the test compound that resulted in complete inhibition of bacterial growth represented the MIC. The MIC of each compound was determined at least twice.

The time-kill curve assay was used to examine the bactericidal effect of 1-pentadecanol. A 30-μl aliquot of a two-day-old culture was inoculated into 3 ml of NYG broth containing the appropriate concentrations of 1-pentadecanol. The initial inoculum was 1×10^6 to 5×10^6 CFU/ml. At 8, 24, and 48 h of incubation, the numbers of viable cells were determined by counting colonies formed from serial 10-fold dilutions plated onto *Actinomyces* agar (BBL). The plates were incubated anaerobically using the Gas Pak Pouch® (BBL) at 37° for 3 days before counting. The effects of 1-pentadecanol were also studied on *Pr. acnes* during the lag and exponential growth phase. The bacterial cultures were prepared as described above, and 1-pentadecanol was added after 0, 8, and 32 h of incubation at appropriate concentrations. At 24 and 48 h after adding the compound, viable counts were determined as described above.

The combination data were obtained by a broth checkerboard method (30). A series of twofold dilutions of one compound was tested in combination with twofold dilutions of the other. The MIC was determined using the same method as described above. All combination studies were carried out at least twice.

SYNTHESIS OF PENTADECANYLDODECANOATE.—To a solution of 1.40 g (7 mmol) of dodecanoic acid and 2.83 g (12 mmol) of 1-pentadecanol in 50 ml of toluene, 5 drops of concentrated H_2SO_4 was added. The mixture was refluxed for 6 h. After the reaction flask was cooled to room temperature, the solution was neutralized with 5% of $NaHCO_3$, washed with saturated aqueous NaCl, dried over $MgSO_4$, and evaporated. The residue was purified by cc on Si gel with *n*-hexane–EtOAc (20:1), yielding 1.52 g (45%) of pentadecanyldodecanoate: mp 42.0°; ir ν max (Nujol) 1740 cm^{-1}; eims *m/z* $[M-1]^+$ 409. ^1H- and ^{13}C-nmr data also support the assignment.

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