

Sphingosines: Antimicrobial Barriers of the Skin

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Among the factors that control the survival of microorganisms on human stratum corneum are skin lipids, including sphingosines. Because the antibacterial spectrum of sphinganine resembles that of cell wall antibiotics, electron microscopy of sphinganine-treated and untreated *S. aureus* was performed; the lipid induced multiple lesions of cell wall, membrane evaginations and loss of ribosomes. However, comparisons of minimal inhibitory concentration of sphinganine for coccal forms and L-forms of *S. aureus*, which lack cell walls, and of the respective dose-related reductions in colony-forming units demonstrated both the susceptibility of L-forms and their superior resistance. Therefore, cell wall lesions are sequelae of a probable membrane reaction. *Candida albicans* was susceptible to sphinganine, sphingosine, dimethylsphingosine, and to a lesser degree, stearylamine. Liquid assays of these lipids against *Trichophyton mentagrophytes*, *T. tonsurans* and *Epidermatophyton floccosum* established their high susceptibility to sphingosine and stearylamine. Sphinganine was the least effective, perhaps due to the presence of L isomers; *T. tonsurans* was the most sensitive. These four lipids were found to be fungistatic, preventing germination and retarding thalli. Antifungal efficacy was confirmed in vitro on stratum corneum. **Key words:** Skin lipids; Microbial ecology; Antibiotics; Normal flora.

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Stratum corneum lipids, particularly sphingolipids, have long been known as crucial elements in establishing the physical and physiological barrier of skin, including the regulation of transepidermal water loss and of percutaneous absorption (1–3). Skin lipids, moreover, are regarded as significant ecological agents, which retard the colonization and infection of mainly gram-positive bacteria and develop the characteristic normal flora of micrococci and coryneforms (4–6). Primary sphingosines comprise 0.5% of skin lipids (7). We have recently discovered that these lipids, which are released free in the stratum corneum during cornification (7), are inhibitory for a wide variety of cutaneous microorganisms (8). Although among mammalian cells sphingosines affect protein kinase C and – by regulating Ca²⁺ release – growth, differentiation, liver function, and inflammation (9), their molecular cytotoxic effect in bacteria has not yet been ascertained. In this report we describe our further in vitro studies of these potent lipids against bacteria and skin fungi and demonstrate their gross lethal mechanism against *Staphylococcus aureus*.

MATERIALS AND METHODS

Lipids

D-sphingosine, D,L-sphinganine [dimethylsphingosine] (erythro and threo isomers), L- α -phosphatidylcholine and stearylamine were ob-

tained from Sigma Chemical Co. (St. Louis, MO, USA), and N,N-dimethyl-sphingosine was purchased from Matreya (Pleasant Gap, PA, USA). These lipids were dissolved in ethanol to prepare stock solutions. Tween 80 (polyoxyethylene sorbitan monooleate) and Tween 20 (polyoxyethylene sorbitan monolaurate) (J. T. Baker Chemical Co., USA), selected for their dual capacity as nutrients and mild non-ionic detergents, were diluted 1/10 in ethanol.

Microorganisms

Trichophyton mentagrophytes, *T. tonsurans*, *Epidermatophyton floccosum*, *Acinetobacter lwoffii*, and *Neisseria meningitidis* were freshly isolated from clinical specimens. *Bacillus subtilis* came from a contaminated agar dish. *Staphylococcus aureus* strain 502A and *Candida albicans* came from our culture collection. We maintained the dermatophytes on potato dextrose agar, *C. albicans* on Sabouraud dextrose agar, and the bacteria on tryptic soy agar, incubating *N. meningitidis* in a candle jar.

Using a modification of the procedure of Reinhardt et al. (10), we attained spore preparations of each of the dermatophytes by scraping mycelia from three to five plates into a sterile 125 ml Erlenmeyer flask filled with glass marbles. A sufficient amount of an antibiotic wash, consisting of 15 mg chloramphenicol, 75 mg cycloheximide, and 25 mg tetracycline (all Sigma) in 250 ml distilled water, was poured into the flask to cover the marbles. After the flask had been swirled moderately for 1 h in a rotary shaker, the liquid was filtered through sterile gauze packed in a 50-ml syringe. We then spun the suspension in a clinical centrifuge for 30 min, collected the supernatant, and inspected the spores by microscopy. Ten-fold dilutions plated on potato dextrose agar provided viable counts.

Liquid assays

The effect of sphingosines on bacteria and *C. albicans* was examined by our previously described procedure (8), which involved 25 μ l of two-fold dilutions of a lipid (control of ethanol alone) mixed with 275 μ l neopeptone (Difco, USA), and 100 μ l of an appropriate ten-fold dilution of an 18-h culture of the microorganism. For testing L-forms (see below), the assay broth included 5% NaCl and the inoculum was taken from a 7 to 10-day old culture incubated at room temperature. Reactions were carried out at 37°C in a shaking water bath for 60 min. Survivors were then cultured on appropriate agar.

We examined the susceptibility of dermatophytes to sphingosines by quantifying thalli developed from spores. Ten ml of Sabouraud dextrose broth (4% glucose and 1% neopeptone) were placed in mycology bottles, giving a depth of 1–1.5 cm. Each received 25 μ l of a lipid dilution and 10 μ l of a spore preparation to provide 20–50 thalli in the ethanol control. Bottles were incubated at room temperature for 7 days and reexamined at 10 days. This assay provided a measure of minimal inhibitory concentration (MIC).

The MIC of sphinganine against the coccal form of *S. aureus* was determined by standard tube dilutions utilizing 25 μ l of lipid, 25 μ l of a 1:1000 dilution of an 18-h culture (giving a final inoculum of 10⁵ colony forming units (CFU)) and 950 μ l of tryptic soy broth (TSB). Inoculum and ethanol controls were included. The comparative MIC of this lipid against the L-form was ascertained in a mixture of 3.7 ml of L broth (TSB with 5% NaCl and 5% horse serum), 25 μ l of sphinganine dilution and 0.3 ml of undiluted broth culture (2 days at 35°C). Control agents were phosphatidylcholine, Tween 20, Tween 80, ethanol only and broth only. Results were recorded after 48 h.

Electron microscopy

Because staphylococci and streptococci become gram negative after lipid treatment and, at high concentrations of lipid, dried and stained

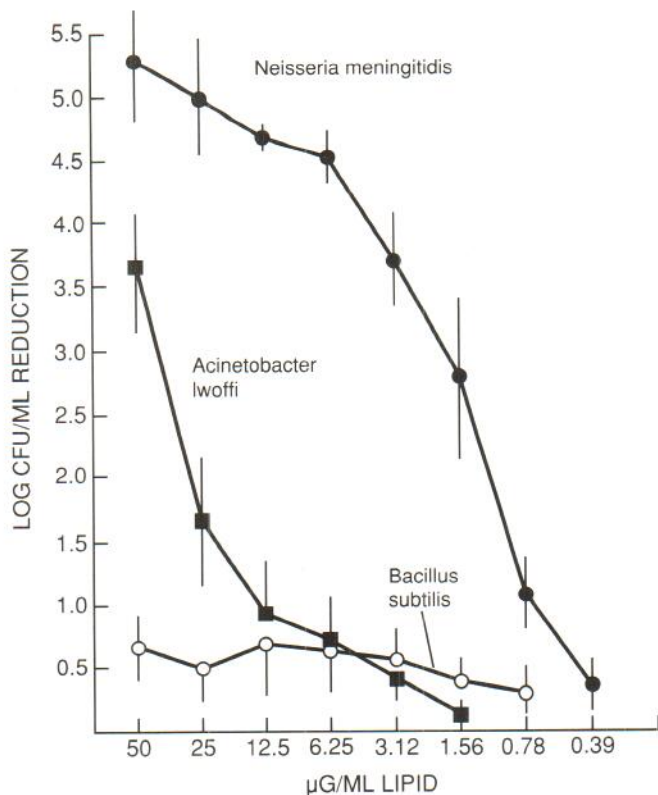


Fig. 1. Effect of sphinganine on various bacteria (mean \pm SD, $n = 4$).

staphylococci are also distorted and granular (8), the cell wall seemed to be the target. For direct inspection, overnight cultures of *S. aureus* were centrifuged and resuspended in filtered 0.01 M tris-buffered neopeptone, pH 6.7, and diluted 1/10 to contain 10^8 CFU/ml. One ml samples were treated with 12.5 μ g sphinganine, or with 25 μ l ethanol alone, for 1 h at 37°C. Following centrifugation and resuspension in filtered tris-buffered normal saline, pH 7.2, specimens were centrifuged and resuspended in filtered phosphate-buffered glutaraldehyde (2.5%) for 30 min. After further centrifugation, treatment with fresh glutaraldehyde continued for 3 h. Post-fixation in OsO_4 , dehydration, embedding and uranyl acetate and lead citrate staining were carried out by standard procedures.

L-forms

For the induction of protoplasts and subsequently L-forms of *S. aureus* strain 502A, we modified the methods of Watanakunakorn (11) and Yabu (12). We cut out blocks containing L colonies and inverted them on fresh agar. Such transferring was repeated until no spontaneous reversion of L-forms to cocci occurred. Stable blocks were then placed in L broth and incubated at room temperature until strands of L-form growth appeared. A series of subcultures improved rate of growth and total mass, which reached 10^7 CFU/ml compared to 10^9 CFU/ml for its parent.

Stratum corneum assay

We examined the antifungal efficacy of sphingosines on skin samples adapting Stanton's method (13). Strips of abdominal skin removed at autopsy from a 38-year-old woman were frozen until use. After having been thawed and cleansed with sterile gauze pads saturated with phosphate-buffered 0.1% Triton X-100 (Eastman Kodak), skin sections were rinsed in sterile water and immersed for 2–3 min in a wash of tetracycline (5 μ g/ml), chloramphenicol (50 μ g/ml) and cycloheximide (0.5 mg/ml) (Sigma). A 5-min bath in hexane followed. We cut 1.5-cm squares of skin and placed sets of five, stratum corneum upward, on sterile filter paper in Petri dishes. Lipids (100 μ g in 25 μ l of solvent vehicle) or solvent alone were applied to the middle cm of each piece. The addition of sterile water to the filter paper was sufficient to

maintain moisture. Having been incubated 24 h at room temperature, the dishes were placed ajar on a rack within a closed water bath heated to 70°C. After exposure for 4–5 min the stratum corneum could be peeled off the epidermis easily. The tissue, either stratum corneum upward or inverted, was then placed upon Bacto-agar (3%) containing the previously given antibiotics. Five to 30 spores of *T. mentagrophytes* or *T. tonsurans* in 1 μ l, delivered by 10 μ l microsyringe, were applied to each corner and center of the medicated zone. Results were recorded after 7 days' incubation at room temperature.

RESULTS

Bacteria

Fig. 1 compares the sphinganine sensitivity of several bacteria that frequently challenge generalities of the gram positive-negative dichotomy and other standards. The narrow antibacterial spectrum as represented here and previously (8) resembles the range of cell wall active antibiotics, such as bacitracin, penicillin, and cephalosporin (14).

Electron microscopy provided further evidence for cell wall susceptibility (Fig. 2A, B). In contrast to the compact ribosome-dense normal staphylococci, the sphinganine-treated cells showed numerous and widely spaced lesions of the cell wall, evaginations of cell membrane and cellular debris through gaps in the wall, and loss or disruption of ribosomes.

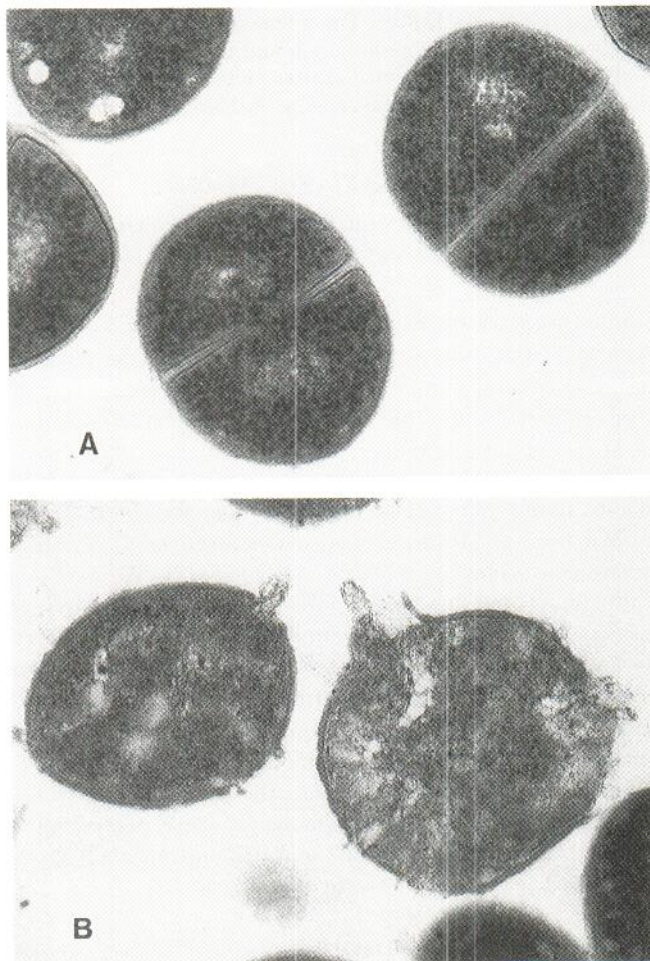


Fig. 2. The lethal effect of sphinganine on *S. aureus*. Length is 0.82 μ m. (A) Untreated cells. (B) Cytopathology includes loss of cell wall, membrane evaginations and leakage.

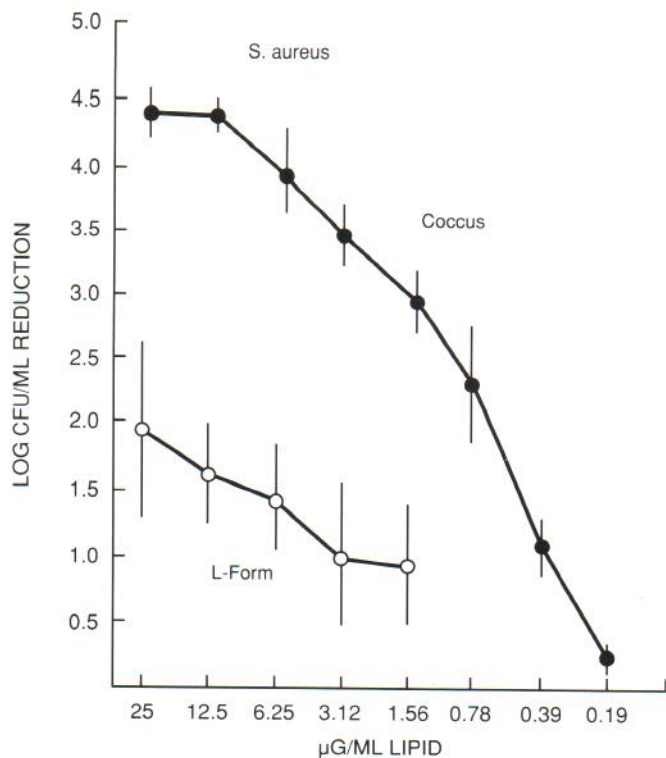


Fig. 3. Susceptibility of L-forms and coccal forms of *S. aureus* to sphinganine (mean \pm SD, $n = 8; 3$).

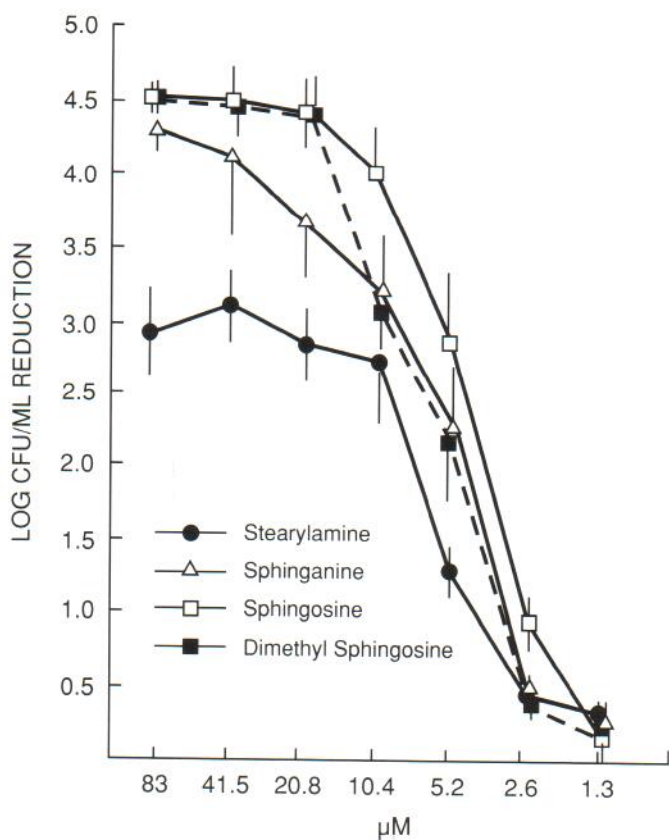


Fig. 4. Comparative activity of sphingosines and stearylamine on *C. albicans* (mean \pm SD, $n = 4-6$).

We could not determine from these photographs whether damage to the cell wall is a primary or secondary effect. However, the induction and cultivation of L-forms, which lack cell walls, permitted us to determine if the wall is a necessary, if not the primary, target. The comparison of MIC quickly answered the question. In our two trials, the growth of parent *S. aureus* was inhibited at 6.25 $\mu\text{g/ml}$. L-forms were inhibited at 12.5 $\mu\text{g/ml}$ with reduced growth at 10 $\mu\text{g/ml}$. Growth was normal at 6.25 $\mu\text{g/ml}$. Although ethanol and phosphatidylcholine had no apparent effect, both Tween 20 and Tween 80 killed, indeed, lysed the L-forms.

Fig. 3, which depicts the reduction of cocci and L-forms after 60 min exposure to sphinganine, provides another perspective of L-form susceptibility. Examining preparations that contained similar concentrations of L-forms, we observed by microscopy only rare intact and dense L-forms in samples taken from the 25 $\mu\text{g/ml}$ assay tubes. In contrast, L-forms were plentiful in the ethanol controls. Therefore, sphinganine-induced lysis had occurred. Nevertheless, in both assays L-forms were more resistant.

Fungi

Fig. 4 compares the activity of sphingosine, sphinganine, dimethylsphingosine, and the similar lipid, stearylamine. Sphingosine consistently was the most lethal and stearylamine had significant but substantially lower activity. The greatest differences among these lipids are noted between 5 and 10 μM .

Table I summarizes the percent reduction in thallus formation in three species of dermatophytes cultured in the presence of various lipids. Although against bacteria sphingosine always proved as the more potent lipid, sphinganine was nearly as lethal. Among the dermatophytes, however, sphinganine could not match the high efficacy of sphingosine. The difference in titer may have been due to the presence of L isomers in the sphinganine preparations, which in this instance may be non-functional. The high potency of stearylamine among dermatophytes contrasts with its relatively poor activity against *C. albicans* and bacteria.

Another distinction from bacterial models is that sphingosines are merely fungistatic. Whether they be inhibited spores or retarded thalli, plating of samples resulted in normal growth of colonies. When 7-day normal thalli were treated with up to 20 $\mu\text{g/ml}$ sphinganine for either 1 h at 35°C or 2 days at 25°C and then plated, colony growth ensued with no morphological differences compared to colonies from an untreated inoculum. In lower concentrations of lipids germination occurred, but only tiny, highly retarded thalli developed that would enlarge slowly, if at all, over an additional week of incubation.

Our qualitative examination of lipid activity in preventing dermatophyte growth on skin essentially confirmed the liquid studies. While growth of *T. tonsurans* and *T. mentagrophytes* was vigorous on solvent sham-treated and untreated skin, we observed no mycelia on samples treated by any of the test lipids. However, these results were restricted to the top surface. Penetration of these lipids through the stratum corneum, i.e. to the spore-inoculated inverted surface, did not occur and unretarded growth was present on all skin samples. Furthermore, we observed no inhibition of dermatophyte growth on

Table I. Effect of lipids on dermatophyte growth in broth from a spore inoculum

Results are mean percent reduction \pm standard deviation ($n = 4$). Figures in parentheses are $\mu\text{g/ml}$ of sphingosine.

	Control thalli	Lipid concentration μM					
		0.415 (0.125)	0.83 (0.25)	1.66 (0.5)	3.32 (1.0)	8.3 (2.5)	16.6 (5.0)
<i>T. mentagrophytes</i>	20.6 \pm 6.5						
Sphingosine	–	–	55.6 \pm 45.1	64.0 \pm 33.0	100	100	
Dimethylsphingosine	–	–	42.0 \pm 38.9	86.1 \pm 27.8	100	100	
Sphinganine	–	–	36.3 \pm 41.0	35.5 \pm 36.7	79.8 \pm 33.6	100	
Stearylamine	–	–	36.6 \pm 18.9	80.0 \pm 40.0	100	100	
<i>E. floccosum</i>	16.0 \pm 5.4						
Sphingosine	–	–	95.6 \pm 5.9	100	100	100	
Dimethylsphingosine	–	–	55.4 \pm 45.5	100	100	100	
Sphinganine	–	–	41.1 \pm 34.0	72.4 \pm 25.6	100	100	
Stearylamine	–	–	88.6 \pm 12.1	100	100	100	
<i>T. tonsurans</i>	47.5 \pm 14.3						
Sphingosine	–	83.9 \pm 32.2	100	100	100	100	
Dimethylsphingosine	–	10.4 \pm 10.0	55.9 \pm 51.8	58.2 \pm 41.9	100	100	
Sphinganine	–	–	–	34.4 \pm 44.6	38.6 \pm 39.9	100	
Stearylamine	–	6.7 \pm 7.9	30.6 \pm 22.9	89.6 \pm 18.8	87.0 \pm 20.1	100	

lipid-treated and normally situated stratum corneum whose top layer was removed by adhesive tape prior to spore inoculation.

Growth on the inverted samples was both more rapid and more robust than that on normally positioned skin. *T. tonsurans* inoculated on top of the stratum corneum developed yellow pigmented mycelia favoring the middle to lower strata. Growth was compact with no fluff. With the stratum corneum inverted, growth ensued at the now upper strata with extensive fluff turning granular. Therefore, hyphal extension increased with depth toward the dermis.

DISCUSSION

Our finding that sphinganine damages the staphylococcal cell wall explains the diminished ability of treated bacteria to adhere to nasal epithelial cells (15). A key observation is that the cell wall lesions are not confined to the growing ring associated with septum formation. The antimicrobial spectrum and the relative resistance of L-forms demonstrate further that the lethal effect of sphinganine is not due to its serving as a non-specific detergent. It is also evident that cell wall lesions, however lethal in their own right, are sequelae of some unknown but also lethal reaction that probably involves membranes.

The differing incidence of dermatophyte species on various skin sites is still poorly understood (16). Specialized characteristics of both microorganism and host tissue environment are probably involved. The distinct susceptibility of the tested fungi to sphingosines (*T. tonsurans* > *E. floccosum* > *T. mentagrophytes*) suggests one possibility. If the content of sphingosines and other antimicrobial lipids vary with the region of skin, then it could help explain the selectivity of species in dermatophytoses.

Our results suggest a gradient of free indigenous sphingo-

sines with the progress of cornification, which contrasts with the reported decrease of sphingolipids from the stratum granulosum to the stratum corneum (17). We tested various solvent formulations in attempts to carry the lipid through the stratum corneum, or at least increase its concentration through the strata, but have yet to find a suitable formulation. The problem is probably related to the limitations of the static skin model, because sphingosine dissolved in ethanol readily passed through the stratum corneum of mice in vivo (18). The physical and chemical relationships of sphingosines in skin bear further study.

Our results continue to demonstrate the wide antimicrobial activity of sphingosines and their ecological role in host defense. Efficacy and toxicological studies in animal models of cutaneous infectious disease and in human volunteers will eventually ascertain the potential of indigenous and derivative sphingosines as novel therapeutic or prophylactic agents.

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