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The synthesis and characterization of analogs of the antimicrobial compound squalamine: 6β-hydroxy-3-aminosterols synthesized from hyodeoxycholic acid

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Analogs of the aminosterol antimicrobial agent squalamine *have been synthesized beginning from hyodeoxycholic acid. After carboxylic acid esterification and oxidation of both alcohol functions to ketones, the A/B ring junction was converted from* cis *to* trans *by acid-catalyzed isomerization. Different polyamines were added to the 3-keto group by reductive amination, yielding both the 3α and 3β addition products. The synthetic products exhibited potent, broad-spectrum antimicrobial activity similar to that of the parent compound. Changing the identity of the polyamine or the stereochemistry of addition has little effect upon antimicrobial activity but appears to change the selectivity of the agents. The analogs are synthesized with high yield from inexpensive starting materials and are promising alternatives to squalamine as potential antibiotics.* © 1996 by Elsevier Science Inc. (*Steroids* **61**:565–571, 1996)

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Introduction

The discovery of low molecular-weight compounds with antimicrobial activity from a wide variety of organisms has led to extensive searches for new agents with potential for development as pharmaceuticals. To date, a large number of such compounds have been isolated and characterized, including peptides, alkaloids, lipids, and sterols.[1,2] These compounds are believed to be an important first line of defense against microbial infection.

Using techniques that had been successfully used to isolate antimicrobial cationic peptides such as magainins, a novel compound with antimicrobial activity was purified from the dogfish shark *Squalus acanthias*.[3] The compound was demonstrated to be the aminosterol 3β-*N*-1-[*N*(3-[4-aminobutyl])-1,3 diaminopropane]-7α,24*R*-dihydroxy-5α-cholestane 24-sulfate by mass spectroscopy,[3] two-dimensional NMR spectroscopy,[4] and by comparison with the synthetic compound.[5–7] This aminosterol antibiotic was named *squalamine* from its source and from its spermidine moiety.

In an effort to relate antimicrobial activity to aminosterol structure, we have been actively synthesizing a wide variety of squalamine analogs from simple bile acids.[8] Ideally, test compounds should be synthesized that differ at a single site from the parent compound, and the biological activities of the test and parent compounds compared to understand structure-activity relationships. However, the preparation of aminosterol analogs is hindered by the unique combination of functional groups and stereocenters on squalamine. The best published synthesis of squalamine[6] was accomplished in 17 steps with 1.9% overall yield, beginning with the expensive starting material 5-cholenic acid. The expense in both time and materials makes the synthesis of close squalamine analogs impractical at this time. As a result, we have concentrated our efforts on bile acid analogs, which do not contain the 24-sulfate or side chain of squalamine, but which can be synthesized with different polyamine substi-

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tutents and with modifications of stereochemistry and substitution of the A and B rings. Therefore, results from bile salt analogs can provide information on how substitutions on the A and B sterol rings affect antimicrobial activity.

Here we report the synthesis of a series of such analogs from hyodeoxycholic acid (Figure 1). Hyodeoxycholic acid was chosen because of the relative ease of conversion of the structure from the 5β or A/B *cis* ring system to the 5α or all-*trans* system via acid catalyzed isomerization of the 6-oxo sterol. The results of this study have generated some understanding of structure-activity relationships for aminosterol antibiotics and allow us to test a published model for aminosterol antimicrobial activity.[9]

Experimental

General methods

Solvents and reagents were purchased as reagent grade and used without further purification. Thin-layer chromatography (TLC) was performed on aluminum plates coated with Merck 5554 Kieselgel 60F. Developed plates were air dried and visualized by charring with 30% sulfuric acid, or by ninhydrin for amines. Column chromatography was performed using silica gel (J. T. Baker). Melting points were determined using a Thomas Hoover melting point apparatus and are uncorrected. Mass spectra were obtained using a VG Analytical ZAB 2-SE high-field mass spectrometer at M-Scan, Inc. (West Chester, PA, USA). Nuclear magnetic resonance spectra were recorded on either a Varian XL-200 or a Bruker AM 400 spectrometer. Deuterated solvents were used for frequency lock, and residual solvent was used as the chemical shift reference. Elemental analyses were performed by Oneida Research Services, Inc.

Methyl hyodeoxycholate (1)

The methyl ester of hyodexoycholic acid was prepared by acid-catalyzed esterification of hyodeoxycholic acid in methanol.[10] Hyodeoxycholic acid (10 g, 25.5 mmol) was added to absolute methanol (200 mL) in a magnetically stirred 500-mL round-bottomed flask, followed by concentrated sulfuric acid (5 mL) dropwise. The reaction was allowed to stir overnight. Dichloromethane (250 mL) was added, and the reaction mixture was washed with sodium carbonate solution (2 × 100 mL), followed by brine (100 mL). The organic layer was then dried over anhydrous sodium sulfate, filtered, and dried under vacuum to yield **1** (10.1 g, 97%).[10]

Methyl 3,6-dioxo-5β-cholan-24-oate (2)

The 3,6-dioxo sterol was prepared by oxidation of **1** with pyridinium chlorochromate.[10] Methyl hyodeoxycholate (**1**, 10.1 g; 25 mmol) was dissolved in dichloromethane (200 mL) in a magnetically stirred ice bath. Pyridinium chlorochromate (33 g, 150 mmol) was added, and the reaction solution was allowed to warm to room temperature. After 8 h at room temperature, the product was the only visible TLC spot. The majority of the methylene chloride was removed under vacuum, and ethyl acetate (250 mL) was added to the flask. The chromium crust in the bottom of the flask was broken up with a spatula, and the contents of the flask were filtered through a celite column. The eluant from the celite column was then reduced in volume under vacuum and filtered through a florosil column (elution with ethyl acetate). The eluant was again reduced in volume to approximately 200 mL, and diethyl ether (100 mL) was added, which was washed with sodium bicarbonate solution (2 × 250 mL) followed by brine (250 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and dried

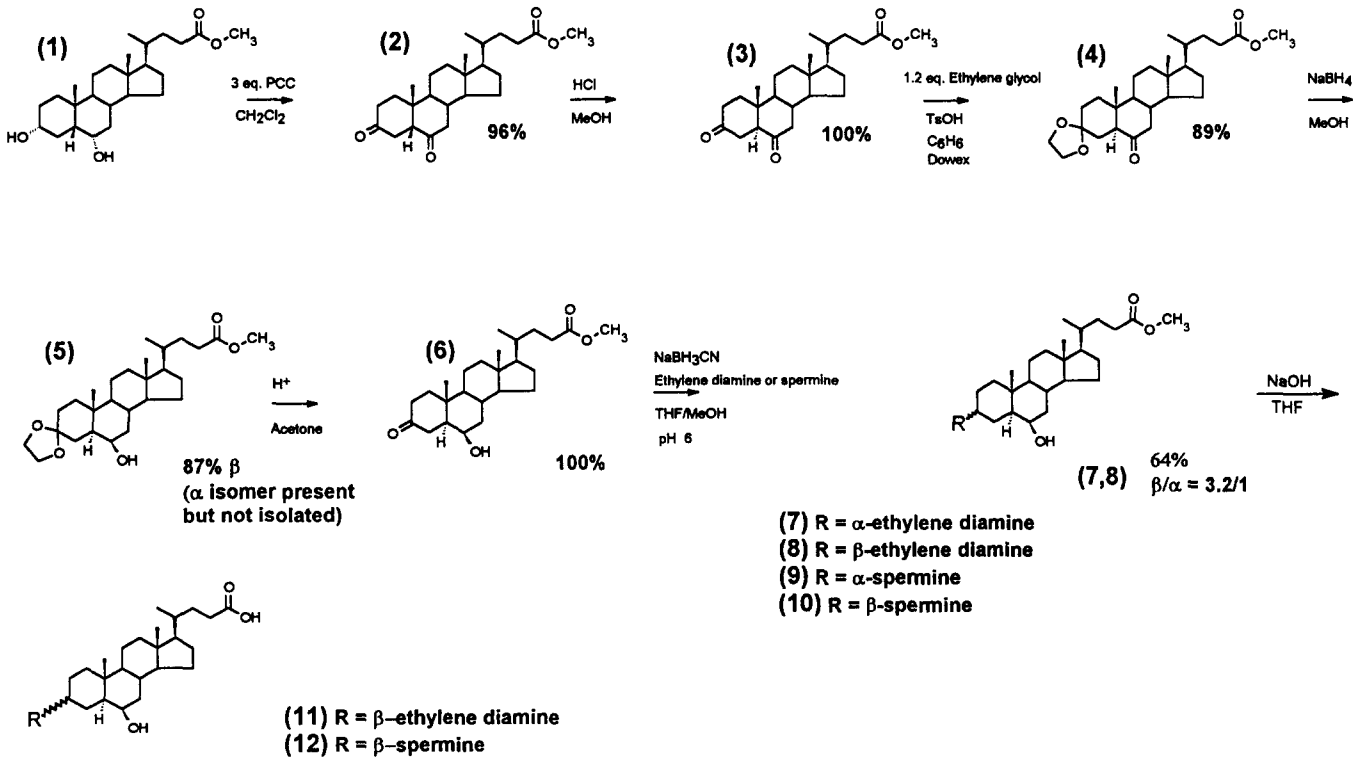

Figure 1 Synthetic scheme for the synthesis of squalamine analogs from hyodeoxycholate. Compound numbers and synthetic yields are indicated in the scheme adjacent to the compounds.

under vacuum. The total yield of **2** was 9.6 g (24 mmol, 96%). **2:** m.p. 126–128°C; ^{13}C NMR (50.3 MHz; $CHCl_3$): δ 210.6, 208.5 (for the C-3 and C-6 ketones, consistent with the results of Demir et al.[10]); ^1H NMR (200 MHz, $CDCl_3$): δ 3.67 (s, 3 H), 0.96 (s, 3 H), 0.71 (s, 3 H).

*Methyl 3,6-dioxo-5α-cholan-24-oate (**3**)*

The 3,6-dioxo-5α sterol was prepared by acid-catalyzed isomerization of the 5β sterol. The 3,6-dioxo-5β sterol **2** (9.6 g, 24 mmol) was added to methanol (250 mL) and tetrahydrofuran (25 mL) to dissolve the sterol completely. Concentrated hydrochloric acid (12.5 mL) was added, and the reaction was allowed to proceed overnight. The solvent was then removed under vacuum to yield 9.6 g (100%) of the 5α sterol **3.** The reaction is similar to that described by Demir et al.,[10] except that the authors used base-catalyzed isomerization with sodium methoxide. **3:** m.p. 150–152°C; ^{13}C NMR (50.3 MHz; $CHCl_3$): δ 211.1, 208.9 (for the C-3 and C-6 ketones, consistent with the results of Demir et al.[10]), 174.4; ^1H NMR (200 MHz, $CDCl_3$): δ 3.66 (s, 3 H), 0.96 (s, 3 H), 0.70 (s, 3 H).

*Methyl 3-dioxolane-6-oxo-5α-cholan-24-oate (**4**)*

The monoprotection of methyl 3,6-dioxo-5α-cholan-24-oate (**3**) was accomplished in several ways. The first attempt involved refluxing **3** (9.6 g, 23.8 mmol) in toluene (250 mL) with ethylene glycol (1.77 g, 28.5 mmol) in the presence of catalytic *p*-toluenesulfonic acid. A Dean Stark trap was used for removal of the toluene-water azeotrope. The reaction was judged to be complete by TLC after approximately 20 min. The reaction was worked up by pouring the toluene over sodium bicarbonate solution (500 mL) and ice slurry. The organic layer was washed with additional sodium bicarbonate (200 mL) and brine (200 mL), dried over anhydrous sodium sulfate, filtered, and dried under vacuum. The crude product was chromatographed on silica gel (4 cm × 25 cm, elution with 33% ethyl acetate in hexanes). The product **4** (8.9 g, 81%) was the second band off the column; the only other product present was the less polar *bis*-dioxolane.

Subsequent attempts substituting benzene for toluene and following the reaction by TLC resulted in greater selectivity. The reaction can be stopped before significant diprotection occurs in the lower boiling solvent. **4:** m.p. 124–126°C; ^1H NMR (200 MHz, $CDCl_3$): δ 4.04-3.93 (m, 4 H), 3.68 (s, 3 H), 0.95 (d, J = 6 Hz, 3 H), 0.78 (s, 3 H), 0.69 (s, 3 H); IR (KBr, cm^{-1}): 2945, 1742, 1709, 1439, 1381, 1313, 1162, 1090; MS (FD): 446 (M^+), 388.

*Methyl 3-dioxolane-6β-hydroxy-5α-cholan-24-oate (**5**)*

The 6β-hydroxy sterol was prepared from the monoprotected diketone by reduction with sodium borohydride. Sodium borohydride (2.5 g, 66 mmol) was added to absolute methanol (200 mL) and stirred for 20–30 min to completely dissolve the borohydride. The 3-dioxolane-6-oxo sterol **4** (5 g, 11 mmol) was dissolved in tetrahydrofuran (10 mL), added to the borohydride in methanol, and allowed to stir overnight. Chloroform (500 mL) was added, followed by washing with distilled water (2 × 200 mL) and brine (100 mL). The organic layer was dried over sodium sulfate, filtered, concentrated under vacuum, and purified by flash chromatography on silica gel (4 cm × 25 cm, elution with 2/1/1 hexanes/ethyl acetate/methylene chloride) to yield **5** (4.35 g, 87%). Alternatively, the crude product can be recrystallized from benzene in hexanes, ethyl acetate in hexanes, or chloroform in hexanes (2×) to yield a product of high purity without the need for column chromatography. **5:** m.p. 164°C; ^1H NMR (200 MHz, $CDCl_3$): δ 4.04-3.93 (m, 4 H), 3.77 (br s, 1 H), 3.66 (s, 3 H), 1.03 (s, 3 H), 0.92 (d, J = 6 Hz, 3 H), 0.69 (s, 3 H); IR (KBr, cm^{-1}): 3533, 2937, 1726, 1438, 1379, 1255, 1191, 1096; X-ray diffraction revealed the expected structure.

*Methyl 3-oxo-6β-hydroxy-5α-cholan-24-oate (**6**)*

The 3-dioxolane was deprotected using acidic acetone solution. The 3-dioxolane-6β-hydroxy sterol **5** (4.0 g, 8.9 mmol) was added to acetone (200 mL) and treated with concentrated HCl (10 mL). After approximately 1 h, the reaction mixture was poured into a sodium bicarbonate solution. The solution was extracted with dichloromethane (3 × 200 mL), which was washed with distilled water (100 mL) and brine (100 mL), dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum to yield the desired product **6** (3.45 g; 100%): ^1H NMR (200 MHz, $CDCl_3$): δ 3.8 (br m, 1 H), 3.69 (s, 3 H), 1.24 (s, 3 H), 0.95 (d, J = 6 Hz, 3 H), 0.74 (s, 3 H); IR (KBr, cm^{-1}): 3447, 2954, 1742, 1707, 1431.

Ethylene diamine and spermine conjugates of methyl 3-oxo-6β-hydroxy-5α-cholan-24-oate

The ethylene diamine analogs were prepared as follows. A magnetically stirred solution of 50:50 methanol/tetrahydrofuran (100 mL) and ethylenediamine (2 mL) was treated with acetic acid to lower the pH to approximately 6. The 3-oxo sterol **6** (1.5 g, 3.7 mmol) was added and allowed to stir for 15 min. Sodium cyanoborohydride (1 g, 16 mmol) was dissolved in 10 mL methanol and added to the reaction vessel. The pH was adjusted to 6 by the addition of acetic acid. The reaction was allowed to stir for 1 h, and the contents of the flask were then poured into a pH 10.5 carbonate buffer/ice slurry (250 mL). The solution was extracted with chloroform (5 × 150 mL). The organic layers were combined, dried over anhydrous sodium sulfate, filtered, dried under vacuum, and purified by flash chromatography on silica gel (4 cm × 25 cm, elution with 8/2/1 chloroform/methanol/isopropylamine) to afford the less polar α-isomer **7** (260 mg, 15%) and the more polar the β-isomer **8** (840 mg, 49%).

7: ^1H NMR (400 MHz, CD_3OD): δ 3.74 (m, 1 H), 3.65 (s, 3 H), 3.53 (m, 1 H), 1.06 (s, 3 H), 0.94 (d, J = 6 Hz, 3 H), 0.74 (s, 3 H); IR (KBr, cm^{-1}): 3426, 2943, 1740, 1590, 1438, 1379, 1258, 1168, 1027; MS (+FAB): 449.5 (M + 1); Analysis calculated for $C_{27}H_{48}N_2O_3$-2HCl-0.7H_2O: C 60.70; H, 9.70; N, 5.24. Found: C, 60.97; H, 9.68; N, 5.34.

8: ^1H NMR (400 MHz, CD_3OD): δ 3.75 (m, 1 H), 3.64 (s, 3 H), 1.02 (s, 3 H), 0.94 (d, J = 6 Hz, 3 H), 0.73 (s, 3 H); IR (KBr, cm^{-1}): 3560, 3366, 3257, 2936, 1726, 1648, 1605, 1438, 1376, 1166, 1047; MS (+FAB): 449.5 (M + 1); Analysis calculated for $C_{27}H_{48}N_2O_3$-0.4 H_2O: C, 71.13; H, 10.79; N, 6.14. Found: C, 71.15; H, 10.71; N, 6.28.

The spermine analogs were prepared by adding the 3-oxo sterol **6** (1.5 g, 3.7 mmol), spermine (2 g, 9.9 mmol), and powdered 3-A sieves (2 g) to a magnetically stirred flask containing anhydrous methanol (100 mL). The pH was adjusted to approximately 6 with acetic acid, the flask was sealed, and the reaction mixture was allowed to stir overnight. Sodium cyanoborohydride (1 g, 16 mmol) in methanol (10 mL) was added, the pH was readjusted to 6 with acetic acid, and the reaction mixture was allowed to stir for 8 h. The workup was identical to that for the ethylene diamine analogs. The crude product was purified by flash chromatography on silica gel (5 cm × 25 cm, elution with 4/5/1 chloroform/methanol/isopropylamine), affording the less polar α-isomer **9** and the more polar β-isomer **10.** The total yield of amino sterol was 1.3 g (58%).

9: ^1H NMR (400 MHz, CD_3OD): δ 3.75 (m, 1 H), 3.65 (s, 3 H), 3.54 (m, 1 H), 1.06 (s, 3 H), 0.95 (d, J = 6 Hz, 3 H), 0.74 (s, 3 H); IR (KBr, cm^{-1}): 3406, 2944, 1740, 1596, 1466, 1168, 1049, 1027; MS (+FAB): 591.4 (M + 1); Analysis calculated for

$C_{35}H_{66}N_4O_3$-4 HCl-1.2 H_2O: C, 55.43; H, 9.62; N, 7.39. Found: C, 55.70; H, 9.15; N, 7.12.

10: ^{1}H NMR (400 MHz, CD_3OD): δ 3.79 (m, 1 H), 3.65 (s, 3 H), 1.06 (s, 3 H), 0.95 (d, J = 6 Hz, 3 H), 0.74 (s, 3 H); IR (KBr, cm^{-1}): 3406, 2944, 1740, 1595, 1459, 1381, 1167, 1051, 1026; MS (+FAB): 591.4 (M + 1); Analysis calculated for $C_{35}H_{66}N_4O_3$-4 HCl-1.2 H_2O: C, 55.43; H, 9.62, N, 7.39. Found C, 55.48; H, 9.03; N, 7.33.

Preparation of free acids 11 and 12

One mmol of aminosterol methyl ester as the free base was weighed into a 25-mL round-bottomed flask. The aminosterol was dissolved in a minimal amount of tetrahydrofuran (2 mL). To the flask was added 1N potassium hydroxide solution (10 mL), and the mixture was magnetically stirred for 1 h. The solution was neutralized with 1N HCl, and the solvent was removed under vacuum. The residue was redissolved in a minimal amount of deionized water and applied to an octadecyl functionalized silica gel column (Aldrich) 2 cm × 10 cm, gradient elution of acetonitrile in 0.2% trifluoroacetic acid in water. The fractions containing aminosterol were pooled and the solvent was removed under vacuum. The aminosterol was redissolved in 0.1N HCl and the solvent was removed under vacuum (2×) to ensure the removal of trifluoroacetate. Benzene was added to the resulting aminosterol hydrochloride salts and evaporated overnight to remove as much water as possible. The ethylene diamine β-amino isomer **11** was not treated with HCl, but isolated as the trifluoroacetate salt.

The ethylene diamine β-isomer **11:** ^{1}H (400 MHz, CD_3OD): δ 3.78 (m, 1 H), 1.06 (s, 3 H), 0.95 (d, J = 6.5 Hz, 3 H), 0.74 (s, 3 H); IR (KBr, cm^{-1}): 3533, 3488, 2941, 1716, 1679, 1615, 1489, 1431, 1191; MS (+FAB): 435.5 (M + 1), 531.5 (likely a trace of the trifluoroacetamide); Analysis calculated for $C_{26}H_{46}N_2O_3$-2 TFA-0.7 H_2O: C, 53.36; H, 7.37; N, 4.15; Found: C, 54.36; H, 7.45; N, 4.40.

The spermine β-amino isomer **12:** ^{1}H NMR (400 MHz, CD_3OD): δ 3.80 (m, 1 H), 1.05 (s, 3 H), 0.95 (d, J = 6.5 Hz, 3 H), 0.73 (s, 3 H); IR (KBr, cm^{-1}): 3406, 2944, 1718, 1637, 1458; MS (+FAB) 577.4 (M + 1); Analysis calculated for $C_{34}N_{64}N_4O_3$-4 HCl-4 H_2O: C, 51.38; H, 9.64; N, 7.05; Found: C, 51.40; H, 8.77; N, 7.01.

Antimicrobial and hemolytic activities

Minimum inhibitory concentrations (MICs) for bacteria and yeast were determined by incubating logarithmic-phase organisms (0.9–1.1 × 10^4 colony-forming units/mL) in trypticase soy broth (Difco) at 37°C for 18–24 h as described previously.[11] The organisms chosen for testing were *Staphylococcus aureus, Escherichia coli* (D31 strain), *Pseudomonas aeruginosa,* and *Candida albicans.*

Results and discussion

Synthesis of squalamine analogs

The general synthetic strategy for the preparation of 6β-hydroxy aminosterol analogs of squalamine from hyodeoxycholic acid in seven to eight steps is shown in Figure 1. Hyodeoxycholic acid methyl ester **1** was oxidized to the A/B *cis* ring dione **2** with pyridinium chlorochromate, which was conveniently converted to the A/B *trans* ring dione **3** by acid catalysis in excellent yield (96% for two steps). Selective protection of the C3 ketone was achieved with ethylene glycol (1.2 equivalents) to afford **4** in 81% yield.

Sodium borohydride reduction and subsequent cleanup by flash column chromatography yielded the 6β-hydroxysteroid **5** with 87% yield. Previous studies of borohydride reduction of similar ketosteroids have demonstrated that the reduction products are predominantly β-alcohols.[12,13] A less polar band from the flash column was analyzed by NMR and found to contain multiple components, including starting material and the 6α-hydroxysteroid. This material was not further characterized. To confirm the stereochemistry of the isolated product, an X-ray crystal structure was acquired, which confirmed the presence of the 6β-hydroxysteroid. The X-ray analysis also confirmed the isomerization of the A/B ring structure, resulting in an all-*trans* steroid.

After deprotection, the ketone **6** was reductively coupled to various polyamines with sodium cyanoborohydride to yield the desired 3-polyaminosterols. After reductive amination, the polyaminosterol products were separated by flash column chromatography. In most cases, it was possible to separate and individually characterize the two isomers at C3. The identity of the column-purified aminosterol was determined by ^{1}H NMR. As determined for squalamine,[4] the hydrogen at C3 for the β-aminosterol has a chemical shift of 3.05 ppm, and demonstrates axial-axial couplings with both the C2 and C4 hydrogens of about 12 Hz. The hydrogen at C3 for the α-aminosterols has a chemical shift of 3.54 ppm and experiences both equitorial-axial and equitorial-equitorial couplings with C2 and C4 hydrogens, resulting in the appearance of a collapsed multiplet appearing as a single broad peak. At 200 MHz, the resonance for the C3 hydrogen of the β-aminosterol is overlapped with other resonances from the polyamine, but the C3 hydrogen resonance for the α-aminosterol is clearly visible.

The compounds reported in this paper were prepared as either the HCl or trifluoroacetate salts (except for **8,** which was isolated as the free base) as solids at room temperature. All of the compounds were soluble in alcohol, and with two exceptions (compounds **8** and **11**), were at least moderately soluble in water. The structures of the isolated 6β-hydroxy aminosterols **7–12,** along with the structure of the natural product squalamine, are shown in Figure 2.

Biological activity

Antimicrobial activities of the synthesized analogs are shown in Table 1, with the reported antimicrobial activities for squalamine[3] shown for comparison. Table 1 demonstrates that aminosterols with potent antimicrobial activity can be synthesized. One analog in this series, the 3β-ethylene diamine methyl ester **8,** has antimicrobial activity almost equal to that of the parent compound squalamine; the compound is more effective on *Candida* but less effective on *Escherichia* and *Pseudomonas.* Most of the other compounds in Table 1 exhibit potent antimicrobial activity as well. The 3β-ethylene diamine compound **11,** which is the free acid form of **8,** is apparently almost inactive.

From Table 1, the following conclusions can be drawn regarding structure-activity relationships for squalamine analogs:

1. The identity of the polyamine chain has only limited importance in the antimicrobial activity in the case of the

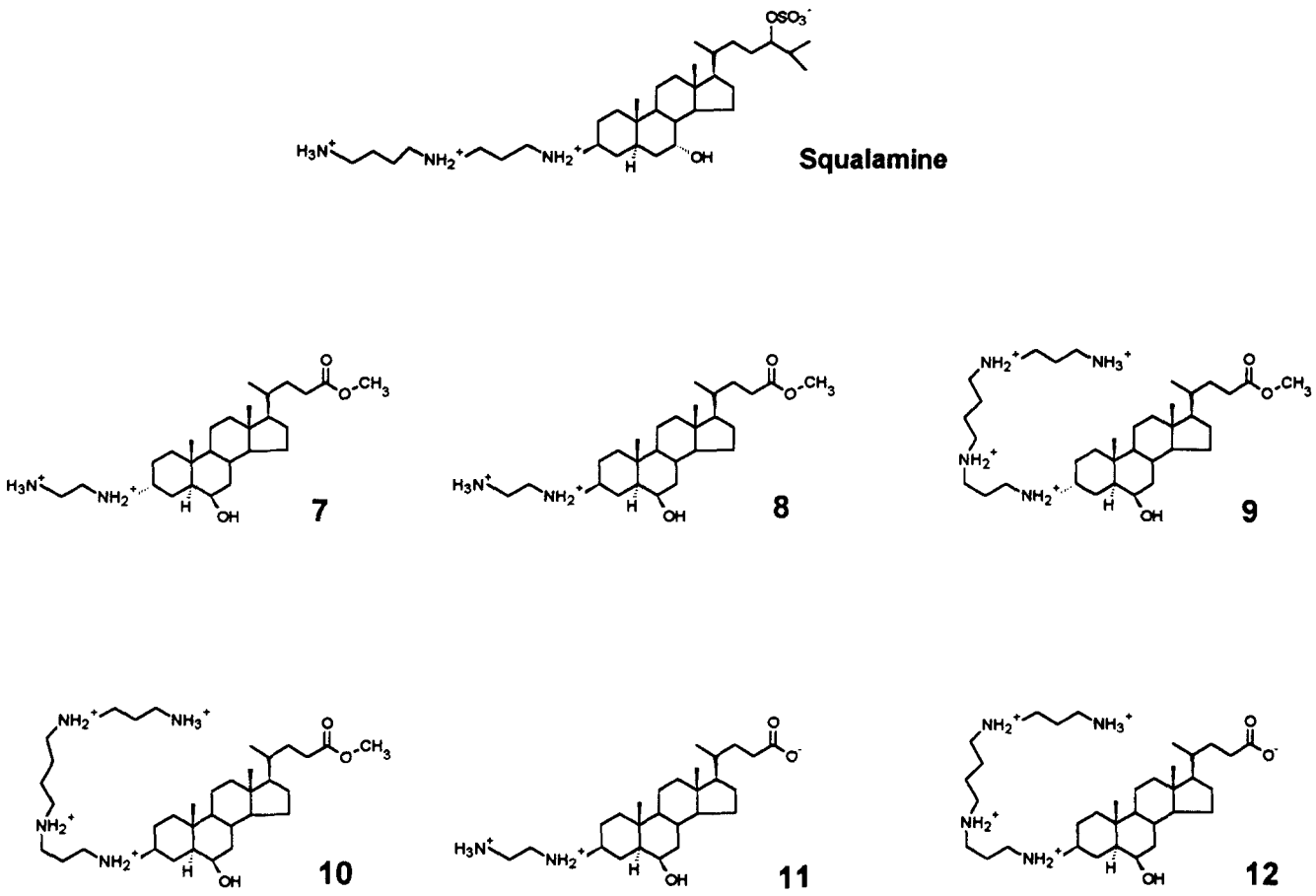

Figure 2 The structure of squalamine and synthetic amino sterols assayed for antimicrobial activity.

esters, but the spermine carboxylic acid **12** is dramatically more active than the ethylene diamine carboxylic acid **11.** None of the 3-ketosterols exhibit antimicrobial activity (data not shown), indicating that the presence of an amine substituent is crucial. The identity of the polyamine may be important in the selectivity of different analogs against different species of microorganisms.

2. From the limited number of analogs reported in Table 1, it appears that the β-analogs are slightly more effective antimicrobial agents than the α-analogs. We note that the β-analogs are preferred synthetically over the α-analogs, and that the spermidine moiety on squalamine is attached with β-stereochemistry at the sterol ring.

3. The methyl esters are more potent antimicrobial agents than the free acids against *S. aureus.* Since squalamine contains a 24-sulfate moiety, the negative charge on that end of the molecule was believed to be important for antimicrobial activity. From this study, that hypothesis is clearly incorrect. However, the negative charge appears to contribute to potency against Gram-negative bacteria, with compound **12** being most potent against *P. aeruginosa.*

The antimicrobial activities are measured by incubating

Table 1 Antimicrobial activities for 6β-hydroxy-5α-3-aminosterol analogs synthesized from hyodeoxycholic acid

	Minimum inhibitory concentrations (μg/mL)			
Squalamine analog tested	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
Squalamine	0.5–1.0	2–4	16	8
3α-Ethylene diamine methyl ester (**7**)	16	32–64	128	8
3β-Ethylene diamine methyl ester (**8**)	1	8–16	64	2–4
3α-Spermine methyl ester (**9**)	2–4	32	128	4
3β-Spermine methyl ester (**10**)	2	32	32	2
3β-Ethylene diamine free acid (**11**)	>256	>256	128	>256
3β-Spermine free acid (**12**)	16	16	8	4

Antimicrobial activities of the naturally occurring aminosterol squalamine are included for comparison.

analogs in the presence of log-phase bacterial cultures at 37°C for up to 24 h. As a result, saponification or transesterification of the esters could possibly occur during the antimicrobial activity. Saponification is unlikely, since the 3β-ethylene diamine methyl ester **8** is a much more potent antimicrobial agent than its saponification product **11.** At this time, transesterification during antimicrobial testing cannot be ruled out. Future syntheses of compounds more similar to squalamine will address the contribution of the side chain and its functional groups on antimicrobial activity.

4. The 6β-hydroxy substituted compounds exhibited antimicrobial activity similar to that of squalamine, suggesting that the absolute positioning of the hydroxyl substituent is not important.

Possible mechanisms for antimicrobial activity

The mechanism for the broad-spectrum antimicrobial activity exhibited by squalamine has not been determined. Because of its structural similarity to the polyene macrolide antibiotic amphotericin B, the antimicrobial activity of squalamine was proposed to be caused by the formation of a transmembrane pore. The spermidine moiety of squalamine would interact with the 24-sulfate, forming a polar face that would line the aqueous interior of a transmembrane pore. The formation of a stable pore would compromise the membrane integrity of the microorganism, resulting in cell death.[14] This hypothesis is supported by the report of the synthesis of a squalamine analog, the spermine amide of 22,23-bisnor-5-cholenic acid-3-sulfate, which exhibits antimicrobial activity against bacteria and yeast.[9]

The activities of the analogs discussed here suggest that the proposed intramolecular interaction of the polyamine with the acidic moiety, although intuitively appealing, may not be important. One of our most active analogs within this series, the 3β-ethylene diamine methyl ester **8,** is incapable of forming a macrocyclic conformation resembling amphotericin B. The ethylene diamine moiety is too short to reach the side chain, and there is no negatively charged group on the side chain available for electrostatic interaction with an amine. None of the methyl esters possess anionic functionality in the side chain, and all have substantial activity.

Bellini and co-workers have synthesized bile salts with amine substitution on the bile salt side chain and have reported that many of these compounds possess antimicrobial activity.[15,16] These compounds have only hydroxyl substituents at C-3 and are incapable of forming the macrocyclic structure proposed as essential for antimicrobial activity. Therefore, based upon the compounds synthesized by Bellini and co-workers, and upon the antimicrobial agents synthesized in this report, we conclude that a macrocyclic structure is not essential for antimicrobial activity.

Electrostatic interactions between the positively charged amines and negatively charged lipid on the surface of bacterial cells certainly contribute to the mechanism of antimicrobial activity. Electrostatic interactions between the amine groups on lysine side chains and negatively charged bacterial phospholipids have been demonstrated to be important in the mechanism of the antimicrobial peptide magainin 2-amide.[17] However, if this were the case for squalamine analogs, one would predict some relationship between antimicrobial activity and the length of the amine substituent, a relationship that is not observed in Table 1.

One possible mechanism for the antimicrobial activity of squalamine and analogs begins with nonspecific electrostatic interactions with bacterial membrane lipids, followed by specific interactions with another component of the bacterial cell membrane. Squalamine has recently been shown to inhibit the sodium-proton antiporter of eukariotic cells (Donowitz et al., manuscript in preparation). The inhibition of a similar antiporter in bacterial cell membranes may be the actual cause of cell death. A specific interaction between a bacterial cell membrane component and the aminosterols is supported by the different potencies of the synthetic squalamine analogs upon different organisms. From Table 1, the most potent analog against *Staphylococcus* and *Escherichia* is the 3β-ethylene diamine methyl ester **8.** However, the 3β-spermine free acid **12** is the most effective against *Pseudomonas,* whereas the 3β-spermine methyl ester is best against *Candida.* If a nonspecific interaction between agent and membrane were responsible for the antimicrobial activities of these agents, one would expect that structural modifications of the agents would either increase or decrease the potency of the agents in all organisms. The selective increases and decreases suggest that the agents may be acting upon a specific site in each organism, which differs slightly from organism to organism. In that way, one agent may bind better to a *Pseudomonas* site than to a *Candida* site.

Squalamine analogs possess no detergent properties

We have considered the possibility that these agents may simply act like cationic detergents. If aminosterols bind to the surface of a negatively charged microbial membrane, the local concentration may increase to disruptive concentrations. Aggregation would then occur, followed by loss of integrity of the plasma membrane and eventual cell death. However, a simple nonspecific interaction between squalamine analogs and microbial plasma membranes does not explain the lack of relationship between polyamine length and activity, the observed differences in specificity between microorganisms for some of our agents, and the very low minimum inhibitory concentrations exhibited by these compounds. Furthermore, we have attempted to measure critical micelle concentrations for several squalamine analogs by dye partitioning. In all cases, the aggregation of squalamine analogs was not observed at concentrations close to the minimum inhibitory concentrations. For these reasons, a detergent-like mechanism for the activity of squalamine analogs is unlikely.

In conclusion, a series of easily prepared aminosterol analogs of squalamine have been synthesized that have antimicrobial potency rivaling that of the parent compound. In particular, the 3β-spermine carboxylic acid **12** has good potency against our four screening organisms (MIC = 4–16 μg/mL). Although the absolute necessity of the anionic group for antibacterial activity is in doubt, further investigation of the nature of the negative charge is clearly warranted. The specific interactions of squalamine with its target are also under active investigation.

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References

1. Zasloff M (1992). Antibiotic peptides as mediators of innate immunity. *Curr Opin Immunol* **4**:3–7.
2. Boman HG (1991). Antibacterial peptides: Key components needed in immunity. *Cell* **65**:205–207.
3. Moore KS, Wehrli S, Roder H, Rogers M, Forrest JN Jr, McCrimmon D, Zasloff M (1993). Squalamine: An aminosterol antibiotic from the shark. *Proc Natl Acad Sci USA* **90**:1354–1358.
4. Wehrli S, Moore KS, Roder H, Durell S, Zasloff M (1993). Structure of the novel steroidal antibiotic squalamine determined by two-dimensional NMR spectroscopy. *Steroids* **58**:370–378.
5. Moriarty RM, Tuladhar SM, Guo L, Wehrli S (1994). Synthesis of squalamine, a steroidal antibiotic from the shark. *Tetrahedron Lett* **35**:8103–8106.
6. Moriarty RM, Enache LA, Kinney WA, Allen CS, Canary JW, Tuladhar SM, Guo L (1995). Synthesis and absolute configuration of squalamine dessulfate. *Tetrahedron Lett* **36**:5139–5142.
7. Pechulis AD, Bellevue FH III, Cioffi CL, Trapp SG, Fojtik JP, McKitty AA, Kinney WA, Frye LL (1995). Total synthesis of squalamine, an anti-infective polyamine. *J Org Chem* **60**:5121–5126.
8. Jones SR (1995). The synthesis and characterization of antibiotic analogs of squalamine. *Biophys J* **68**:A99.
9. Sadownik A, Deng G, Janout V, Regan SL, Bernard EM, Kikuchi K, Armstrong D (1995). Rapid construction of a squalamine mimic. *J Am Chem Soc* **117**:6138–6139.
10. Demir AS, Sabol MR, Jeganathan A, Dolence EK, Watt DS (1987). Synthesis of biological markers in fossil fuels 5. A synthesis of 5α-cholan-24-oic acid. *Org Prep Proced Int* **19**:197–208.
11. Zasloff M (1987). Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* **84**:5449–5453.
12. Rosenkranz G, Velasco M, Sondheimer F (1954). Steroids. LVII. Cycloethylene ketals of androstane-3,6,17-trione. Synthesis of androstan-3-one-6β, 17β-diol. *J Am Chem Soc* **76**:5024–5026.
13. Wheeler DMS, Wheeler M (1972). Reductions of steroidal ketones. In: Fried J, Edwards JA (eds), *Organic Reactions in Steroid Chemistry*. Van Nostrand Reinhold Company, New York, pp. 61–110.
14. Stadler E, Dedek P, Yamashita K, Regan SL (1994). Amphotericin B mimics: A sterol-based ionophore. *J Am Chem Soc* **116**:6677–6682.
15. Bellini AM, Quaglio MP, Guarneri M, Gavazzini G (1983). Antimicrobial activity of cholane compounds: Cholic and deoxycholic acids derivatives (Part I). *Eur J Med Chem Chim Ther* **18**:185–190.
16. Bellini AM, Quaglio MP, Guarneri M, Gavazzini G (1983). Antimicrobial activity of cholane compounds: Cheno and ursodeoxycholic acids derivatives (Part II). *Eur J Med Chem Chim Ther* **18**: 191–195.
17. Matsuzaki K, Sugishita K-I, Fujii N, Miyajima K (1995). Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. *Biochemistry* **34**:3423–3429.