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Squalamine: An aminosterol antibiotic from the shark

(antibacterial/antifungal/steroid/spermidine/defense)

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ABSTRACT **In recent years, a variety of low molecular weight antibiotics have been isolated from diverse animal species. These agents, which include peptides, lipids, and alkaloids, exhibit antibiotic activity against environmental microbes and are thought to play a role in innate immunity. We report here the discovery of a broad-spectrum steroidal antibiotic isolated from tissues of the dogfish shark *Squalus acanthias*. This water-soluble antibiotic, which we have named squalamine, exhibits potent bactericidal activity against both Gram-negative and Gram-positive bacteria. In addition, squalamine is fungicidal and induces osmotic lysis of protozoa. The chemical structure of the antibiotic 3β-*N*-1-{*N*-[3-(4-aminobutyl)]-1,3-diaminopropane}-7α,24ζ-dihydroxy-5α-cholestane 24-sulfate has been determined by fast atom bombardment mass spectroscopy and NMR. Squalamine is a cationic steroid characterized by a condensation of an anionic bile salt intermediate with spermidine. The discovery of squalamine in the shark implicates a steroid as a potential host-defense agent in vertebrates and provides insights into the chemical design of a family of broad-spectrum antibiotics.**

Animals must defend themselves against environmental microbes if they are to survive. Multiple mechanisms of host defense against microbes have been described such as the array of humoral and cellular responses of the classical vertebrate immune system and less-specific physical and chemical barriers. Over the past several years, an increasing number of low molecular weight antibiotic substances, believed to play a role in defense against environmental microbes, have been isolated from diverse species of animals. These molecules include peptides (1–3), lipids (4, 5), and alkaloids (6–8).

In the course of our studies exploring the diversity of antibiotics from animal sources, we have surveyed tissues from a number of animal species (9, 10). We focused our search for antibiotic substances on the gastrointestinal tract of various animals after the recent discovery of peptide antibiotics in the gut of frogs (11), pigs (12, 13), mice (14), and humans (34). In the course of our survey, we discovered that stomach extracts of the shark *Squalus acanthias* exhibited potent antimicrobial activity, prompting efforts to purify and identify the responsible molecule.

In this report we describe the isolation, structural determination, and characterization of a water-soluble cationic steroid from the shark that exhibits potent antimicrobial activity against fungi, protozoa, and both Gram-negative and Gram-positive bacteria. This molecule is shown to be an unusual adduct of spermidine with an anionic bile salt intermediate that, to our knowledge, is without precedent in vertebrates. We have named the aminosterol "squalamine," derived from the genus *Squalus* and its chemical structure as an amine.

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MATERIALS AND METHODS

Purification of Squalamine. *Squalus acanthias* sharks were captured off the New England coast. The shark stomach tissue (400 g) was frozen immediately after dissection, pulverized in liquid nitrogen, and extracted with 5 vol of 60% (vol/vol) acetonitrile in 1% trifluoroacetic acid. After centrifugation, the supernatant was lyophilized and resuspended in 250 ml of 0.1% trifluoroacetic acid. Next, the sample was extracted by a modification (15) of the Folch method (16). The aqueous phase was recovered, lyophilized, resuspended in 30 ml of H_2O, and then loaded onto a 45 × 5 cm Bio-Gel P-30 (Bio-Rad) gel-filtration column in 0.1% trifluoroacetic acid/20% acetonitrile. The fractions were dried under vacuum, resuspended in water, and assayed as described (11). Active fractions (see Fig. 1*a*, fractions 133–153) containing low molecular weight molecules (M_r = 500–1000) were pooled and applied to a C_{18} reversed-phase HPLC column (4.6 × 220 mm, Aquapore OD-300; Applied Biosystems). After flushing the column extensively with buffer A (0.1% trifluoroacetic acid in H_2O) and allowing the absorbance to return to baseline, material was eluted with a linear gradient of 0–60% buffer B (0.08% trifluoroacetic acid in acetonitrile) in buffer A in 45 min at a flow rate of 1 ml/min. Fractions were dried, resuspended in water (100 μl), and assayed. Fractions containing the main peak of activity were pooled and then loaded onto a strong cation-exchange HPLC column (4.6 × 200 mm, 5 μm, 300A, polysulfoethyl aspartamide; Poly LC, Columbia, MD). After loading the sample, the column was washed extensively with buffer C (5 mM KH_2PO_4, pH 3/25% acetonitrile), and the absorbance was allowed to return to baseline. The column was eluted with a gradient of 0–60% buffer D (5 mM KH_2PO_4, pH 3/25% acetonitrile/1 M NaCl) in buffer C in 45 min at 1 ml/min. The fractions were dried and assayed as above. The fractions containing the main peak of activity were then pooled and loaded onto a C_4 reversed-phase HPLC column (4.6 × 25 mm, 5 μm, Vydac; The Separations Group). The C_4 column was developed with the same buffers used for the C_{18} column (buffers A and B). The column was washed with buffer A until no absorbance was detected and then developed with a gradient of 0–30%

Abbreviations: FAB, fast atom bombardment; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-propanesulfonate; DMSO, dimethyl sulfoxide; amu, atomic mass unit(s); CPF, caerulein precursor fragment; TMS, tetramethylsilane.

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buffer B in buffer A in 20 min, followed by 30–50% buffer B in buffer A in 30 min.

Other *Squalus acanthias* tissues (≈50 g of liver, gallbladder, spleen, testes, gill, and intestine) were extracted as described above and purified through the C_{18} HPLC stage. Antimicrobial activity was detected in each tissue, and one reversed-phase fraction from each tissue was analyzed by fast atom bombardment (FAB) mass spectroscopy. The yield from the stomach was determined by spotting several dilutions onto a TLC plate and comparing the ninhydrin staining to a spermidine standard curve. An estimate of the yield of squalamine from other tissues was made by comparing the antimicrobial activity of reversed-phase fractions to the activity from the stomach.

Antimicrobial and Hemolytic Assays. Minimal inhibitory concentrations for the bacteria and yeast were determined by incubating logarithmic-phase organisms (0.9–11 × 10^5 colony-forming units/ml) in 0.5× trypticase soy broth (TSB; Difco) at 37°C for 18–24 h. Samples for the assay were adjusted to pH 6.6 with sodium acetate buffer. Spermidine hydrochloride, taurolithocholic acid 3-sulfate, melittin, holothurin, and ampicillin were purchased from Sigma. CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate dihydrate} was purchased from Aldrich. Conessine was purchased from Aldrich Rare Chemicals Division. Magainin-II amide and caerulein precursor fragment (CPF)-amide were made by Magainin Pharmaceuticals (Plymouth Meeting, PA) The CPF peptide used in the assay has the following sequence GFGSFLGKALKAALKIGANAL-GGSPQQ-NH_2 (17). The assay of minimal disruptive concentration (the concentration at which physical disruption of the protozoa occurred) has been described (18). The hemolytic assay has also been described (9).

FAB Mass Spectroscopy. The methods for the FAB analysis have been described (11).

NMR Spectroscopy. Squalamine solution (5 mM) in dimethyl sulfoxide (DMSO) (310 K) was examined at 600 MHz on a Bruker AM 600 spectrometer. The conditions utilized were 16 scans, 2.5-kHz sweep width, 4096 data points, 2-sec repetition time, and 90° pulse flip angle. The chemical shifts are referenced to tetramethylsilane (TMS) using DMSO at 2.5 ppm as a secondary reference.

The ^{13}C spectrum of a squalamine solution (5 mM) in ^2H_2O at pH 2 (310 K) was examined at 100.62 MHz on a Bruker AM 400 spectrometer. The conditions utilized were 43,000 scans, 25-kHz sweep width, 32.768 data points, 0.76-sec repetition time, and 30° pulse flip angle. Chemical shifts are referenced to an external capillary of dioxane at 67.5 ppm.

RESULTS

Purification of Squalamine. Squalamine was isolated initially from the stomach of the dogfish by a modification of a procedure used (11) in our isolation of peptide antibiotics from the stomach tissue of the frog *Xenopus laevis*. Antimicrobial activity was assayed using *Escherichia coli* D31 and purified through a series of steps involving organic extraction, size-exclusion chromatography, and reversed-phase and cation-exchange HPLC (Fig. 1). At the final stage of purification (Fig. 1*d*), only one molecular species could be detected by TLC using iodine, ninhydrin, and charring detection methods (data not shown).

Determination of the Structure of Squalamine. The chemical structure of squalamine was determined by FAB mass spectroscopy and NMR. The FAB mass spectrum, in the positive-ion mode, yielded a simple fragment pattern, characterized by a molecular ion of 628 atomic mass units (amu) (Fig. 2*a*).

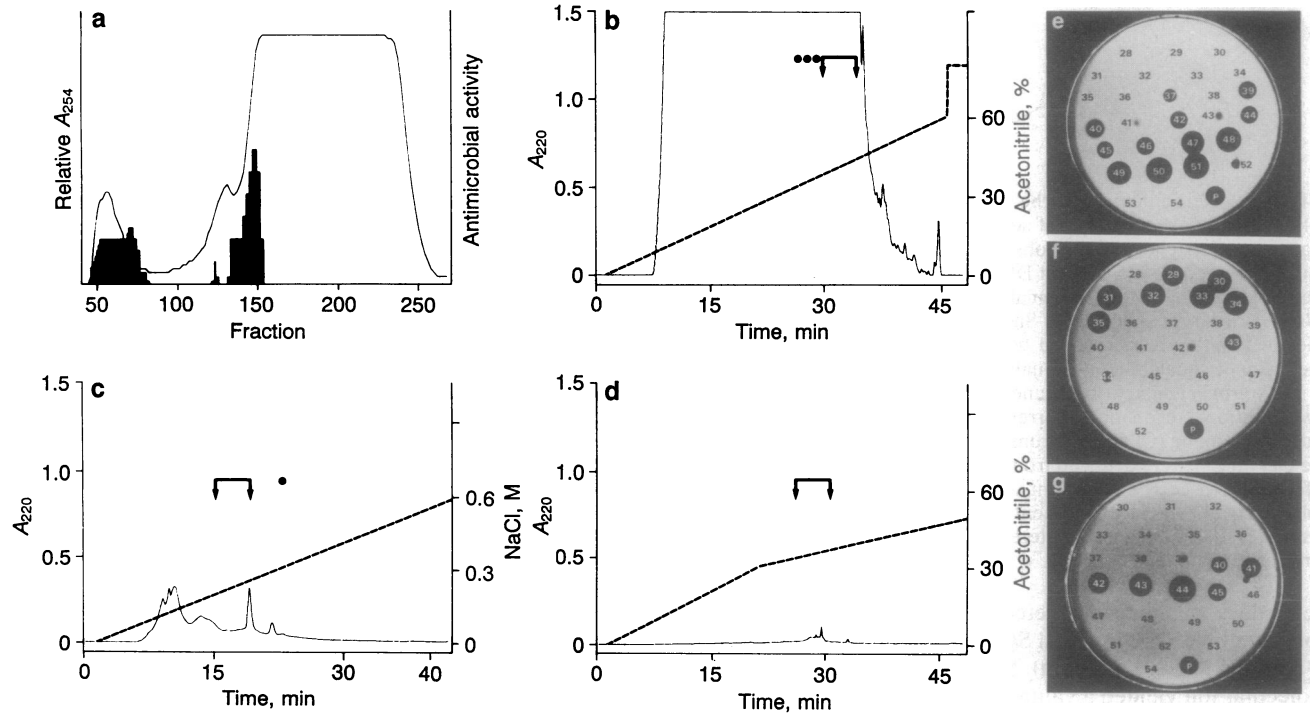

FIG. 1. Isolation of the aminosterol squalamine from dogfish stomach. (*a*) P-30 gel-filtration column. Solid line shows A_{254}. Histogram indicates relative antimicrobial activity against *E. coli* D31. (*b*) C_{18} reversed-phase HPLC. (*c*) Strong cation-exchange HPLC. (*d*) C_4 reversed-phase HPLC. (*e*) Antimicrobial activity of C_{18} reversed-phase HPLC fractions. (*f*) Antimicrobial activity of ion-exchange HPLC fractions. (*g*) Antimicrobial activity of C_4 reversed-phase HPLC fractions. (*b–d*) The solid line shows A_{220}. The dashed line shows the buffer gradient. The bracket with the arrows indicates the major antimicrobial peak that was pooled for the next step of purification or analysis. The dots indicate secondary peaks of antimicrobial activity against *E. coli* D31. (*e–g*) Numbers refer to fraction numbers. (*e*) Fractions 28–54 correspond to an elution time of 21–35 min. (*f*) Fractions 28–52 correspond to an elution time of 15–28 min. (*g*) Fractions 30–54 correspond to an elution time of 22–35 min. *P* = 200 ng of PGLa, a peptide antibiotic from *Xenopus laevis* (19), as a control.

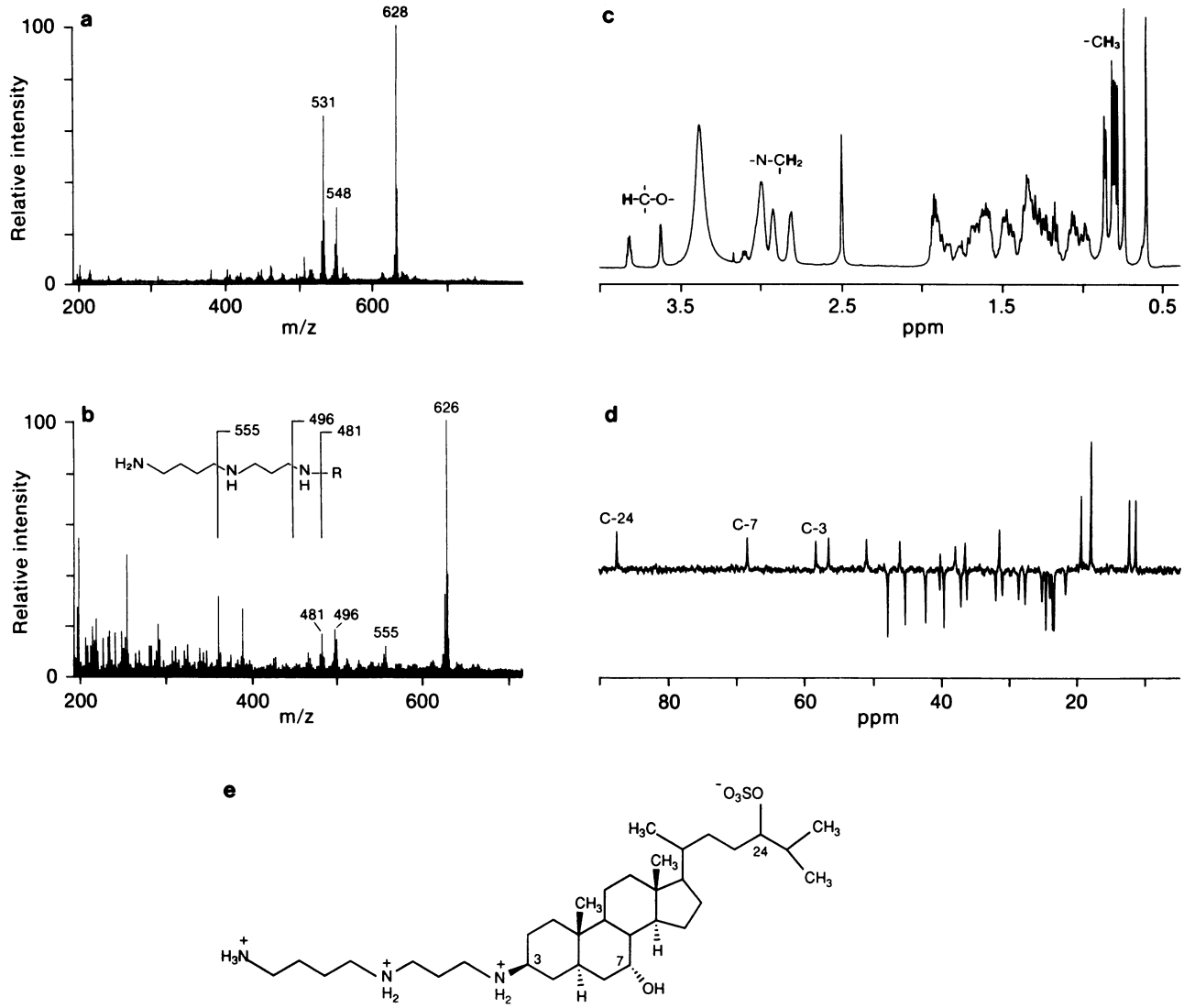

FIG. 2. Determination of the structure of the shark antibiotic squalamine. (*a*) FAB mass spectroscopy of squalamine, positive-ion mode. (*b*) FAB mass spectroscopy of squalamine, negative-ion mode. (*Inset*) Spermidine substituent with lines indicating fragments of the molecule that are consistent with the observed ions. R = 7α,24ζ-dihydroxy-5α-cholestane 24-sulfate. (*c*) Proton NMR spectrum of squalamine. A squalamine solution (5 mM) in DMSO (310 K) was examined at 600 MHz. Signals at 0.6 and 0.75 ppm are characteristic of the C-18 and C-19 methyl groups of a steroid. Signals between 0.8 and 2.0 ppm include other steroid protons. Signals between 2.8 and 3.1 ppm correspond to the -NCH_2 protons of spermidine. Signals between 3.6 and 3.9 ppm correspond to the HC-OH proton of C-7 and the HC-OSO_3 proton of C-24. The chemical shifts are referenced to TMS using DMSO at 2.5 ppm. (*d*) ^{13}C distortionless enhancement by polarization transfer (DEPT) NMR spectrum of squalamine. A squalamine solution (5 mM) in ^2H_2O, pH 2 (310 K), was examined at 100.62 MHz. Chemical shifts are referenced to an external capillary of dioxane at 67.5 ppm. Peaks have been phased such that CH and CH_3 are positive and the CH_2 resonances are negative. Two additional resonances, corresponding to the two quarternary carbons (at 36.15 and 43.18 ppm), visible on the one-dimensional ^{13}C composite pulse decoupling (CPD) spectrum, have been suppressed by the pulse sequence. The position of the spermidine on C-3, the OSO_3 on C-24, and the OH on C-7 were deduced from analysis of two-dimensional total correlated spectroscopy, correlated spectroscopy, and nuclear Overhauser effect spectroscopy experiments. The complete assignments were obtained from the studies above as well as heteronuclear multiple quantum coherence 1J and 3J experiments (35). (*e*) Structure of the antimicrobial aminosterol squalamine isolated from the dogfish shark *Squalus acanthias*. Assignments and stereochemistry of substituents were determined by NMR studies and FAB mass spectroscopy. The fully ionized form of squalamine is shown.

Secondary species were detected at 548 amu and 530 amu, consistent with the loss of a SO_3 ion, either alone (80 amu) or in addition to water (18 amu). Measurement of the mass of the molecular ion yielded a value of 628.4739, consistent with an elemental composition of $C_{34}H_{66}O_5N_3S_1$.

The chemical structure of squalamine in DMSO and H_2O was studied by ^1H and ^{13}C NMR (Fig. 2 *c* and *d*). Squalamine exhibited a proton spectrum characteristic of a steroid (0.5–2.5 ppm) and a molecule of spermidine (3.0–3.3 ppm). Two-dimensional correlation spectroscopy permitted assignment of all signals and established that the steroid contained an α-AB ring junction. Positions of the free OH on C-7, the sulfate on C-24, and the spermidine on C-3 were established unambiguously by the complete analysis of the total correlated spectroscopy, correlated spectroscopy, and nuclear Overhauser effect spectroscopy two-dimensional spectra (35). The specific amino group of the spermidine involved in coupling to the steroid nucleus was deduced by NMR analysis and confirmed by the mass spectrum obtained in the negative-ion mode (Fig. 2*b*), which revealed ions of mass 481, 496, and 555, consistent with fragments generated by the loss of all or portions of the spermidine moiety (Fig. 2*b inset*).

The Structure of Squalamine. The deduced structure of the antibiotic isolated from shark stomach is presented in Fig. 2*e*.

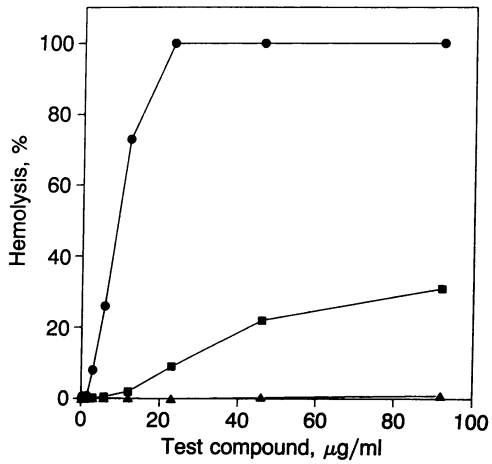

FIG. 3. Hemolytic activity of squalamine. Hemolytic activity of melittin, squalamine, and magainin-II amide. Circles, mellittin; squares, squalamine; triangles, magainin-II amide. Samples diluted in H_2O were added to a 10% suspension of human erythrocytes in phosphate-buffered saline. After incubation of 37°C for 10 min, the samples were centrifuged, and the A_{350} of the supernatant was measured to determine the extent of hemolysis. Addition of Triton X-100 defined 100% hemolysis. The assay results for compounds that were not included in the graph are as follows: CPF at 92 μg/ml showed 3% hemolysis and holothurin caused 14% hemolysis, whereas CHAPS, taurolithocholic acid 3-sulfate, ampicillin, spermidine, and conessine all caused <0.5% hemolysis.

The molecule has a steroid ring structure like cholestanol. Spermidine is coupled to the C-3 position, replacing the usual steroid hydroxyl or ketone group. In addition, C-7 and C-24 are hydroxylated, and the C-24 hydroxyl is sulfated. The stereochemistry of the junction between rings A and B in squalamine (5-α) is seen commonly in bile alcohols of many species of fish (20, 21). C-24 hydroxylation of the cholesterol side chain occurs widely in fish, reptiles, and amphibia and is modified by sulfation in some of these vertebrates (20, 21). Consistent with the deduced structure of squalamine, its antibiotic activity was demonstrated to be resistant to boiling and protease treatment (data not shown).

A review of the chemical data base (Chemical Abstracts, STN International Database, on-line search, May 1991) leads us to conclude that squalamine represents the first reported example of a steroid to which spermidine is covalently coupled. A review of the chemical structures of naturally occurring aminosterols reveals the similarity of squalamine to several cationic steroids isolated from medicinal plants used in the treatment of intestinal parasitic infections (22). For example, squalamine resembles the antiparasitic aminosterol chonemorphine from the Indian plant *Chonemorpha macrophylla* (23).

Squalamine Is a Broad-Spectrum Antibiotic. Squalamine exhibits a broad-spectrum antibiotic activity, including potent microbicidal activity against Gram-negative and Gram-positive bacteria, fungi, and protozoa. *Paramecium caudatum* appears to undergo osmotic lysis, swelling, and eventually bursting, as observed after exposure to magainin (18).

Because of its structural resemblance to steroidal detergents, squalamine was assayed for hemolytic activity against human erythrocytes. Although squalamine exhibits some hemolytic activity, this activity occurs at higher concentrations than observed for nonselective membrane disruptive amphipathic molecules, such as mellitin (Fig. 3). Furthermore, squalamine exerts antibiotic activity at concentrations below which erythrocyte disruption is observed; in contrast, the hemolytic and antibiotic activities of mellitin occur within a similar concentration range (Table 1 and Fig. 3).

The activity of squalamine was compared with several other related molecules including two antibiotic peptides from *Xenopus laevis*, magainin-II (9) and CPF (2). Squalamine compares in antibacterial potency to ampicillin, a broad-spectrum antibiotic used therapeutically. Conessine, an alkaloid of plant origin used therapeutically as an antiparasitic agent (22, 24), exhibits antifungal and antiprotozoan activity but lacks antibacterial activity, demonstrating that potent antibiotic activity is not a trivial property of all cationic steroids. Holothurin, a steroid glycoside from the sea cucumber (25), exhibits modest antiprotozoan activity but is without antibacterial or antifungal activity, demonstrating that surface-active steroids of the saponin family are not comparable to squalamine in antimicrobial activity. Spermidine is inactive as an antibiotic in the concentration range studied, as are the anionic bile salt taurolithocholic acid 3-sulfate and the synthetic zwitterionic steroidal detergent CHAPS. These data suggest that the biological activity of squalamine results from the synergistic combination of an anionic bile salt with spermidine, each of which independently exhibits considerably less antibiotic activity than squalamine.

The importance of the amine substitution on the steroid ring of squalamine for its antibiotic activity is supported by recent studies. The synthesis of a common bile acid (e.g., deoxycholic acid) containing a basic group has been shown to impart the steroid with unexpected antibiotic activity (26–28). The potency and antibiotic spectrum of these synthetic amino-bile acids was shown to be dependent on the position and nature of the hydroxyl and amino substituents (28). In particular, addition of an amino or ethylamino group onto the C-3 position of the deoxycholate ring system markedly en-

Table 1. Antimicrobial spectrum of squalamine and related molecules

Sample	Antimicrobial activity (MIC), μg/ml							
	E. coli (25922)	*Pseudomonas aeruginosa* (27853)	*Staphylococcus aureus* (29213)	*Streptococcus faecalis* (29212)	*Proteus vulgaris* (13315)	*Serratia marcescens* (8100)	*Candida albicans* (14053)	*Paramecium caudatum*
Squalamine	1–2	4–8	1–2	1–2	4–8	>125	4–8	4–8
CHAPS	>500	>500	>500	250–500	>500	>500	>500	>260
Taurolithocholic acid 3-sulfate	>500	>500	>500	>500	>500	>500	>500	>260
Spermidine	>500	>500	>500	250–500	>500	>500	>500	>260
Melittin	8–16	16–31	8–16	4–16	16–31	>250	16–31	2–4
Magainin-II amide	31–62	31–62	>250	>250	125–250	>250	125–250	33–65
CPF-amide	8–16	8–31	8–16	31–62	62–125	>125	62–125	4–8
Conessine	>500	>500	>500	>500	>500	>500	31–62	16–33
Holothurin	>500	>500	>500	>250	>500	>500	>500	130–260
Ampicillin	2–4	62–125	<1	<0.25	8–16	4–62	>125	>65

American Type Culture Collection numbers are in parentheses. MIC, minimal inhibitory concentration.

hances its antibiotic activity; however, these derivatives exhibit a spectrum primarily against Gram-positive bacteria (28). Amine substitutions also dramatically reduce both hepatic clearance of these modified bile acids from the bloodstream and their secretion into bile (29) and might be expected to alter their systemic biodistribution compared to bile salts.

The Tissue Distribution of Squalamine. The tissue distribution of squalamine was determined by using the extraction procedure described for stomach on freshly obtained tissues of *Squalus*. Squalamine could be detected in many tissues of the shark. Liver and gallbladder, the organs in which bile salts are synthesized and stored for secretion into the gastrointestinal tract, are the richest sources identified (4–7 μg/g of tissue). However, both the spleen and the testes of this animal are also relatively rich sources of squalamine (each containing ≈2 μg/g). The stomach (1 μg/g), the gills (0.5 μg/g), and the intestine (0.02 μg/g) yielded lesser amounts. Thus, it is clear that squalamine is also present in organs that are not engaged in the synthesis of bile salts for digestive functions. We are not yet certain whether a single organ, such as the liver, is the principal site of synthesis of squalamine, with subsequent widespread distribution taking place, or whether squalamine is synthesized in many different tissues. It is also possible that squalamine is not synthesized by the shark, but rather, derives from an exogenous source present in the shark's food chain.

DISCUSSION

In this report we have described the discovery of squalamine, a broad-spectrum antibiotic isolated from the dogfish shark. This unusual steroid, representing an adduct between spermidine and an anionic bile salt intermediate, would appear to be the product of an unknown biochemical pathway involving the condensation of spermidine with a steroid. To our knowledge, squalamine represents a previously undescribed class of naturally occurring antibiotics of animal origin. Of the known low molecular weight antibiotics of defined structure, cationic peptides form the largest group (30). Lipids with antibiotic activity, present on the skin of many species of animals, are represented principally by sphingolipids (5), fatty acids (4, 5), and both fatty acid esters (31) and O-alkyl ethers (32, 33) of polyhydric alcohols. Alkaloids form a third chemical class and have been isolated from amphibian skin secretions (6–8). They include metabolites of histidine and tryptophan, such as spinaceamine and bufotenine, and more complex molecules such as samandarine (7).

The presence of squalamine in many tissues of the shark leads us to speculate that squalamine may serve as a systemic antimicrobial agent in this animal, but at this time we have no direct evidence to support this speculation. The biosynthetic pathway of squalamine, its role in host defense, and its expression after injury and infection are yet to be investigated.

Note Added in Proof. We have recently succeeded in chemically synthesizing squalamine and have demonstrated that the synthetic compound is identical to the natural substance in its physical and biological properties (R. Moriarty, S. Tuladhar, L. Guo, S.W., K.S.M., and M.Z., unpublished observations). A full report of the synthesis of squalamine will be published elsewhere.

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