ORGANIC LETTERS

2001
Vol. 3, No. 3
437−440

Bogorol A Produced in Culture by a Marine *Bacillus* sp. Reveals a Novel Template for Cationic Peptide Antibiotics

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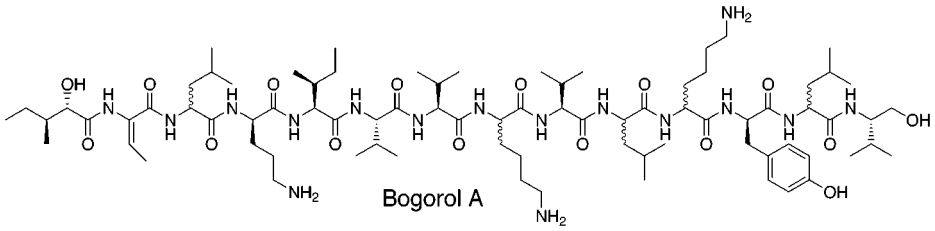
Received November 30, 2000

ABSTRACT



Bogorol A (1), a novel peptide antibiotic active against MRSA and VRE, has been isolated from cultures of a marine *Bacillus* sp. collected in Papua New Guinea. The structure of bogorol A was elucidated by a combination of spectroscopic analyses and chemical degradation. Bogorol A illustrates a new structural template for "cationic peptide antibiotics".

The emergence of widespread antibiotic resistance in the common gram-positive human pathogens *Staphylococcus aureus*, *Enterococcus faecalis*, and *E. faecium* has created a medical problem of crisis proportions.[1] The situation is most acute in nosocomial (hospital-acquired) infections. *S. aureus* is an especially virulent pathogen that is responsible for a wide range of infections including pimples, pneumonia, osteomyelitis (bone marrow infection), endocarditis (heart valve infection), and bacteremia (systemic blood infection). Enterococci are responsible for urinary tract, wound, intra-abdominal, and pelvic infections.

Vancomycin is the antibiotic of last resort for treating enterococci infections that are resistant to all other antibiotics. Initially, it was believed that vancomycin resistance in enterococci would not develop in clinical settings because it is the most complex acquired resistance property known, involving up to seven genes.[1b,2] However, in the past few years, vancomycin-resistant enterococcal strains (VRE) have begun to emerge and there are no effective antibiotics currently available for such organisms.[3] Methicillin-resistant *S. aureus* (MRSA) strains that are resistant to all antibiotics except vancomycin account for roughly 20% of all *S. aureus* isolates in hospital settings.[1b] There is great concern that it is just a matter of time until nosocomial MRSA strains that

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10.1021/ol006942q CCC: $20.00
Published on Web 01/17/2001

are also vancomycin resistant emerge. Infections caused by these bacteria would be untreatable.

In response to the antibiotic-resistance crisis, there has recently been renewed interest in discovering novel classes of antibiotics that have different mechanisms of action.[4] One broad class of compounds attracting a lot of attention are the "cationic peptide antibiotics" [5] that are widespread in nature where they play an important role in the innate immune systems protecting living organisms from microbial infections.[5d,e] These peptide antibiotics are particularly appealing because they kill bacteria quickly, in part by physically disrupting cell membranes, and as a consequence they appear to avoid rapid emergence of resistance.[6]

Antibacterial cationic peptides have two distinguishing features. First, their structures usually contain no more than a single negatively charged amino acid and an excess of basic amino acids (lysine, arginine, ornithine, etc.) resulting in a net positive charge of at least +2 (often +4, +5, or +6) even at neutral pH. Second, these peptides fold in three dimensions so that they have a polar hydrophilic face composed of the positively charged basic amino acid residues and a nonpolar hydrophobic face composed of lipophilic amino acid residues. Four distinct folding types that encompass these characteristics have been identified. They are β sheet structures stabilized by disulfide bridges, α-helices, extended helices (polyprohelices) with a predominance of one or more amino acids, and loop structures. Cationic peptide antibiotics typically show broad spectrum activity against Gram positive bacteria, Gram negative bacteria, and even fungi with MIC's in the range 1−8 μg/mL against both susceptible and antibiotic-resistant pathogens. Protegrin and magainin, cationic peptide antibioitcs isolated from pigs[7] and frogs,[8] respectively, have served as lead structures for the development of analogues that are in various stages of preclinical or clinical evaluation.

As part of a program designed to identify new antibiotics produced by marine microorganisms,[9] it was found that a bacterium isolated from a tropical reef habitat produced the novel "cationic peptide antibiotic" bogorol A (**1**) and a series of very minor analogues in culture. The marine bacterial isolate PNG276,[10] tentatively identified as a *Bacillus laterosporus* by analysis of cellular fatty acids and 16S RNA, was obtained from the tissues of an unidentified tube worm collected off of Lolata Island, Papua New Guinea. It was cultured as lawns on trays of solid tryptic soy agar supplemented with NaCl. After 5 days, the cells were scrapped off the agar surface and extracted exhaustively with MeOH. Bioassay guided fractionation of the concentrated combined MeOH extract by sequential application of solvent partitioning, Sephadex LH 20, reversed-phase flash chromatography, and reversed-phase HPLC gave a pure sample of bogorol A (**1**) (3 mg from 30 g wet cell weight).

Bogorol A (**1**) gave a $[M + H]^+$ ion in the HRFABMS at *m/z* 1584.08500, appropriate for a molecular formula of $C_{80}H_{142}O_{16}N_{16}$. Examination of the 500 MHz ^{1}H, ^{13}C, COSY, HOHAHA, HMQC, HMBC, and ROESY data for bogorol A showed that the molecule was a peptide and identified several of the amino acid residues including tyrosine, valine, leucine, isoleucine, lysine, and ornithine. However, limited resolution in the 500 MHz ^{1}H NMR spectrum precluded a complete structure elucidation. Therefore, bogorol A (**1**) was converted into its acetylated derivative **2** by reaction with acetic anhydride in pyridine at room temperature, and NMR data on this compound was acquired at 800 MHz.

Acetylated bogorol A (**2**) gave a $[M + 1 + H]^+$ ion in the HRFABMS at *m/z* 1837.15212, appropriate for a molecular formula of $C_{92}H_{154}O_{22}N_{16}$ indicating the formation of a hexaacetate. Hydrolysis of the hexaacetate **2** at 100 °C for 72 h with 6 N HCl and examination of the pentafluoropropionamide isopropyl ester derivatives of the liberated amino acids via chiral GC analysis confirmed the presence of L-valine, L-leucine, D-leucine, L-isoleucine, D-tyrosine, L-lysine, D-lysine, and D-ornithine.

Six new methyl resonances that were not in the ^{1}H NMR spectrum of the natural product **1** appeared in the 800 MHz ^{1}H NMR spectrum of acetylated bogorol **2**. Three of them had chemical shifts (δ 1.75, 1.76, and 1.77) appropriate for acetamides presumed to be on the side chains of lysine and ornithine residues, one had a chemical shift (δ 2.23) appropriate for a phenol acetate which had to be on the tyrosine residue, and two of them had chemical shifts (δ 1.95 and 2.07) typical of aliphatic acetate esters that had to be associated with fragments other than the amino acids identified by hydrolysis.

COSY and HMBC data revealed that one of the acetate esters was incorporated in a 2-acetoxy-3-methylpentanoyl unit (Figure 1A). HMBC correlations observed between a carbonyl resonance at δ 170.1 and both a methine proton resonance at δ 4.75 (d, J = 5.6 Hz) and an acetate methyl resonance at δ 2.07 identified the secondary acetate functionality in this fragment. The methine resonance at δ 4.75 showed a single COSY correlation to a methine at δ 1.85 that was further correlated to a methyl resonance at δ 0.88 (d, J = 7.2 Hz) and to a pair of geminal methylene proton resonances at δ 1.15 and 1.50, which were both correlated

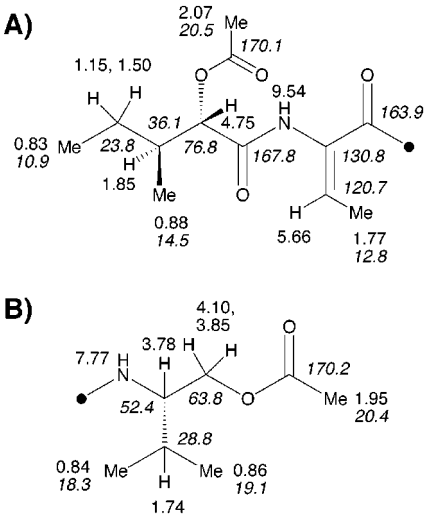

Figure 1. Partial structures in bogorol A hexaacatate (**2**).

to a methyl resonance at δ 0.83. This set of COSY correlations demonstrated that the acetoxy methine carbon was attached to a *sec*-butyl group and a HMBC correlation observed between the methine resonance at δ 4.75 and a ^{13}C resonance at δ 167.8 provided evidence that it was also attached to a carbonyl.

An olefinic methine resonance at δ 5.66 (q, $J = 7.2$ Hz) and an olefinic methyl resonance at δ 1.77 (d, $J = 7.2$ Hz) in the ^{1}H NMR spectrum of **2** could be assigned to a 2-amino-2-butenoic acid residue (Figure 1A). HMBC correlations were observed between the olefinic methine (δ 5.66) and the α carbon (δ 130.8) and carbonyl (δ 163.9) resonances in this dehydroamino acid. A NH resonance at δ 9.54 showed HMBC correlations to both the carbonyl (δ 163.9) and β carbon (δ 120.7) resonances of the 2-amino-2-butenoic acid residue and also to the carbonyl resonance (δ 167.8) of the 2-acetoxy-3-methylpentanoyl residue, demonstrating that the two units were linked via an amide bond as shown in Figure 1A. The olefinic resonance at δ 5.66 showed a strong NOESY correlation to the NH resonance at δ 9.54, indicating that the olefin had the *E* configuration.

To determine the absolute configuration of the 2-hydroxy-3-methylpentanoic acid residue in **2**, the four possible stereoisomers were synthesized as a mixture by reduction of racemic 3-methyl-2-oxopentanoic acid with sodium borohydride and separated into the diastereomeric pairs (*R*,*R*/*S*,*S* and *R*,*S*/*S*,*R*) via normal phase HPLC. Comparison with commercially available samples of D (2*R*,3*R*) and D-*allo* (2*R*,3*S*) 2-hydroxy-3-methylpentanoic acids via chiral GC analysis of their isopropyl esters identified the configurations of the individual enantiomers. 2-Hydroxy-3-methylpentanoic acid was obtained from **2** by ether extraction of the 6 N HCl hydrosylate. Chiral GC comparison of its isopropyl ester with the synthetic samples showed that bogorol A contained (2*S*,3*S*)-2-hydroxy-3-methylpentanoic acid as illustrated in Figure 1.

A pair of geminal methylene proton resonances at δ 3.85 and 4.10 and a methyl resonance at 1.95 all showed HMBC correlations to a carbonyl resonance at δ 170.2, demonstrating that the second aliphatic acetate ester in **2** involved a primary alcohol. COSY correlations were observed from the methylene resonances (δ 3.85, 4.10) to a methine at δ 3.78 that was correlated to another methine at δ 1.74, which was in turn correlated to two methyl resonances at δ 0.86 and 0.84, indicating that the acetylated primary alcohol was part of a valinol residue as shown in Figure 1B. The HMBC, HSQC, and TOCSY data for **2** were in complete agreement with a valinol substructure. Chiral GC analysis comparison of the pentafluoropropionyl derivatives of the 6 N HCl hydrolysis products from **2** with the pentafluoropropionyl derivatives of authentic D and L valinol confirmed the presence of L-valinol.

From a detailed analysis of the 800 MHz 2D NMR data (Table 1 in Supporting Information), it was also possible to identify three valine, three leucine, one isoleucine, one phenolacetylated tyrosine, one side-chain acetylated ornithine, and two side-chain acetylated lysine residues in hexaacetyl bogorol A (**2**). These 11 amino acids together with the 2-acetoxy-3-methylpentanoyl, 2-amino-2-butenoyl, and acetylated valinol fragments had a total elemental composition that was consistent with the HRFABMS and ^{13}C APT data obtained for **2**. It was apparent from the nature of the fragments in **2** that the molecule was a linear peptide with the acetylated valinol residue at the C-terminus and the N-acylated 2-amino-2-butenoyl residue (Figure 1, A and B) at the N-terminus.

A partial sequence for the 11 amino acids in the central core of the hexaacetate **2** could be determined from the FABMS data as shown in Figure 2A. One ambiguity that could not be resolved by the FABMS analysis was the precise location of the isobaric leucine and isoleucine residues. The NOESY spectrum of **3** contained an uninterrupted ladder of $NN_{(i,i+1)}$ N*H* correlations that is typical of α-helix secondary structure in linear peptides.[11] As shown in Figure 2B, this series of NOESY correlations provided support for the amino acid sequence that was determined from the FABMS data and also unambiguously resolved the issue of situating the isoleucine residue. Therefore, the constitution of bogorol A hexaacetate (**2**) is as shown in Figure 2.

The absolute configurations of the valine, isoleucine, tyrosine, ornithine, 2-hydroxy-3-methylpentanoic acid, and valinol residues are as shown in Figure 2. Hydrolysis showed that two of the leucines are L and one is D, while there is one L and one D lysine residue; however, their precise locations in bogorol A could not be determined by spectroscopic analysis. Efforts to resolve this final issue via total synthesis are ongoing in our laboratory.

Bogorol A (**1**) was tested for antibacterial activity against a panel of human pathogens. It showed good activity against MRSA (MIC 2 μg/mL) and VRE (10 μg/mL), moderate activity against *Escherichia coli* (35 μg/mL), and no activity against *Stenotrophomonas maltophilia* (>200 μg/mL),

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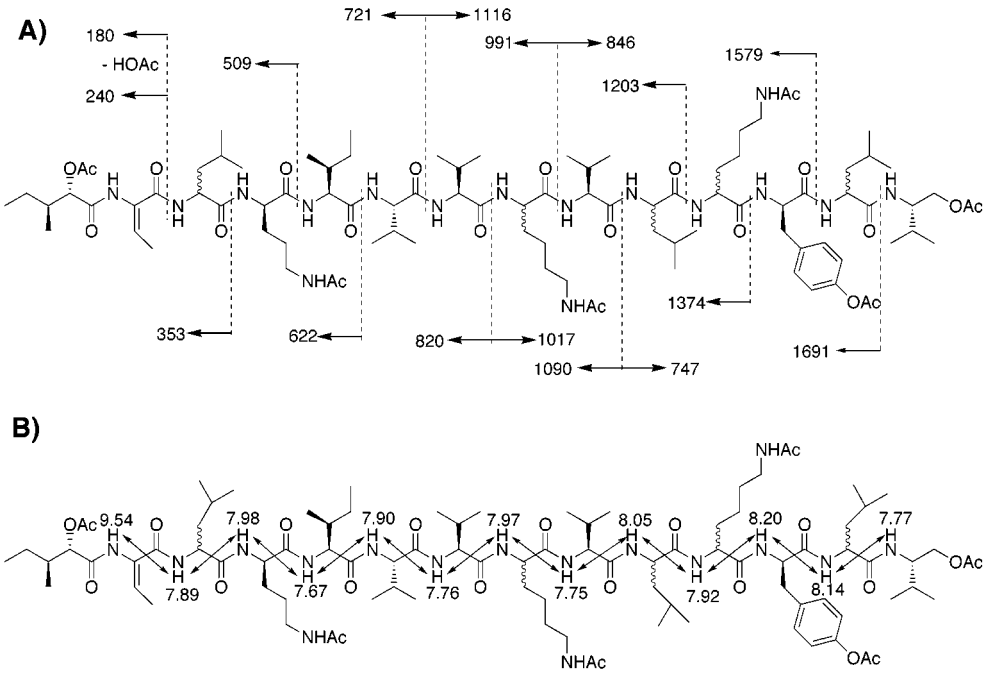

Figure 2. (A) FABMS fragmentation pattern for bogorol A hexaacetate (**2**). (B) Observed NOESY correlations between N*H* resonances.

Berkholderia cepacia (>200 μg/mL), drug resistant *Pseudomonas aeruginosa* (>200 μg/mL), or *Candida albicans* (>200 μg/mL).

Bogorol A (**1**) contains a number of structural features that are typical of nonribosomal peptides. These include reduction of the C-terminal residue to give valinol, transformation of a N-terminal isoleucine to 2-hydroxy-3-methylpentanoic acid, incorporation of four D amino acids, and the presence of a dehydroamino acid. As a consequence of the C- and N-terminal modifications, all of the potentially charged residues in bogorol A reside in the interior of the linear peptide chain. To the best of our knowledge there are no previously known linear "cationic peptide antibiotics" with both C-terminal aminol and N-terminal α-hydroxy acid modifications. Bogorol A shows selective and relatively potent activity against MRSA and VRE, and since it represents a new "cationic peptide antibiotic" template it is an attractive lead structure for SAR optimization via either combinatorial synthesis or combinatorial biosynthesis.

Acknowledgment. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada. We thank Helen Wright for technical assistance.

Supporting Information Available: NMR spectra and table of NMR assignments for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL006942Q