JOC Article

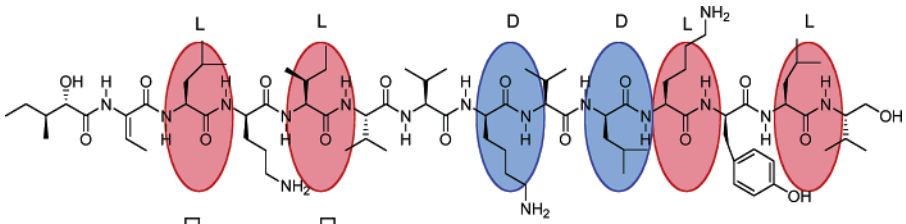
The Bogorol Family of Antibiotics: Template-Based Structure Elucidation and a New Approach to Positioning Enantiomeric Pairs of Amino Acids

Todd Barsby,[†,‡] Kaoru Warabi,[†] Dan Sørensen,[†,§] William T. Zimmerman,[†,|] Michael T. Kelly,[⊥] and Raymond J. Andersen*[,†]

Departments of Chemistry and Earth & Ocean Sciences, University of British Columbia (UBC), 2036 Main Mall, Vancouver, British Columbia, V6T 1Z1, Canada, and Ocean Pharmaceuticals, Inc., 4156 H Street Road, Blaine, Washington 98230

randersn@interchange.ubc.ca

Received March 28, 2006



Bogorol A (**1**)

Bogorol B (**2**)	valine	no change
Bogorol C (**3**)	valine	valine
Bogorol D (**4**)	methionine	no change
Bogorol E (**5**)	methionine sulfoxide	no change

The sequence positions of D and L Leu and Lys residues in bogorol A (**1**) have been defined by a simple and novel approach that utilizes small amounts of sample and focuses on detecting the order in which amino acids are liberated from the parent peptide during acid-catalyzed hydrolysis. This technique builds on a previously established relationship between the steric and electronic features of amino acids and their predilection for acidic liberation from polypeptides via dipeptides. The results, which complete the structure of bogorol A, have been confirmed by traditional degradation experiments. Utilizing the knowledge of the structure of bogorol A (**1**) as a template, we rapidly elucidated the structures of bogorols B−E (**2**−**5**) via analysis of ESI-MS and ESI-MS/MS data and GC analysis of degradation products. The bogorol cationic peptide antibiotics contain a number of unusual structural features, which include the reduction of the C-terminal residue to valinol, an N-terminal residue of 2-hydroxy-3-methylpentanoic acid, the incorporation of four D amino acids, and the presence of a dehydroamino acid. Bogorols show selective and relatively potent activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp., as well as moderate activity against *Escherichia coli*.

Introduction

North Americans gained a 10-year increase in their life expectancy during the 20-year period following the discovery of antibiotics and their introduction into clinical use to combat infections.[1] Most of the structural classes of antibiotics used in human medicine today had been discovered by the end of the 1950s,[2,3] and the principal advances over the intervening years have come from second- and third-generation semisynthetic

[†] Departments of Chemistry and Earth & Ocean Sciences, UBC.
[‡] Present address: Faculty of Science, University of Ontario Institute of Technology, 2000 Simcoe Street North, Oshawa, Ontario, L1H 7K4.
[§] Present address: Merck Frosst Center for Therapeutic Research, Merck Frosst Canada Ltd., 16711 Trans Canada Hwy, Kirkland, Québec, H9H 3L1.
[|] On leave from DuPont Crop Protection, Stine-Haskell Research Center, Newark, DE 19711.
[⊥] Ocean Pharmaceuticals, Inc.

10.1021/jo060667p CCC: $33.50
Published on Web 07/08/2006

modifications to existing drugs.[2,3] The chronic widespread use in hospital, agricultural, and other commercial settings of a select number of antibiotic classes that act via a few modes of action set the stage for the antibiotic resistance crisis that currently exists.[4] Important pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* spp. (VRE), which were previously relegated to hospital settings, are now emerging in the general community, making them a serious widespread threat to human health.[5,6] New drug discovery efforts are urgently required to bolster our therapeutic armory against infectious diseases.

"Cationic peptide antibiotics",[7] which are an integral part of the innate immune systems protecting living organisms from microbial infections, are currently attracting a lot of attention as possible new-generation clinically useful antimicrobial agents.[8−10] 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 stimulating the rapid emergence of resistance.[7] 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 a least +2 (often +4, +5, or +6) even at neutral pH. Second, these peptides can fold in three dimensions so that the positively charged basic amino acid residues form a polar hydrophilic face while the lipophilic amino acid residues form a nonpolar hydrophobic face (i.e., they are amphipathic).

The importance of small peptides and proteins to life has driven the development of techniques and instrumentation specifically designed to meet the challenges of elucidating their structures.[11] However, some peptides present unusual structural features that confound the more automated methods of structure elucidation.[12,13] A specific problem encountered when working with peptides that contain both D and L enantiomers of a particular amino acid is the sequence positioning of each enantiomer.[14−17] This problem is frequently left unsolved because large amounts of the natural product are required for classical partial digestion and subsequent fragment identification and the degradation process is tedious and experimentally challenging. New approaches to the problem of sequence positioning of D and L amino acids using small quantities of the natural product are needed.

The marine bacterial isolate PNG-276, identified as *Brevibacillus laterosporus* by the analysis of 16S RNA, was obtained from tissues of an unidentified tube worm collected off the coast of Loloata Island, Papua New Guinea. Crude extracts of *B. laterosporus* cultures grown on solid agar showed broad-spectrum antibiotic activity against the human pathogens MRSA, VRE, *Mycobacterium tuberculosis*, *Candida albicans*, and *Escherichia coli*. Initial bioassay-guided fractionation resulted in the isolation of the loloatins A−D and the basiliskamides A and B.[18,19] Although potent antibiotics, the presence of the loloatins and basiliskamides did not account for the Gram-negative (*E. coli*) activity in the crude extract. A subsequent assay-guided fractionation using *E. coli* as the test organism resulted in the isolation of the linear cationic peptides, bogorols A−E (**1**−**5**). The structure elucidation of bogorol A (**1**) using MS and 800 MHz NMR data was reported earlier;[14] however, the sequence positioning of D and L Leu and Lys residues was left unresolved. Herein we report the application of a relatively simple and general procedure based on peptide hydrolysis for the prediction of the sequence positioning of the D and L Leu and Lys residues in bogorol A (**1**), an experimental confirmation of this procedure, and the use of bogorol A (**1**) as a template for the rapid structure elucidation of the minor bogorol analogues B−E (**2**−**5**) (Figure 1).

Results and Discussion

Sequence Positions of the D and L Leu and Lys Residues in Bogorol A (1). The absolute configurations of the 14 residues that comprise **1** were assigned previously.[14] However, the configurations of specific Leu and Lys residues in the sequence were not resolved. During the structure elucidation of **1**, it was observed that acid hydrolysis performed for short time periods (24 h) gave only partial hydrolysis. GC analysis of the partial hydrolysate showed no Ile residue and very low Val levels relative to its abundance in the parent peptide. Of greatest interest was the observation that the relative abundance of the individual enantiomers of the D/L Leu and D/L Lys pairs in the short-term hydrolysate was not stoichiometric, which suggested a kinetic approach to determining the configurations of Leu and Lys residues at specific locations in the linear peptide.

Partial acid hydrolysis of proteins is known to occur with a degree of selectivity dictated by steric and electronic factors.[20] In particular, amide linkages to Val and Ile carbonyls are found to be the most resistant to hydrolysis. The initial stage of protein hydrolysis with strong acid and high temperatures mainly generates dipeptides, which are relatively resistant to further hydrolysis due to the presence of positively charged ammonium groups. Eventually the dipeptides, according to their constitution,

(1) McDermott, W.; Rogers, D. E. *Johns Hopkins Med. J.* **1982**, *151*, 302−312.
(2) Dax, S. L. *Antibacterial Chemotherapeutic Agents*; Blackie Academic & Professional: London, 1997; p 396.
(3) Labischinski, H.; Johannsen, L. In *Drug Discovery from Nature*; Grabley, S., Thiericke, R., Eds.; Springer: Berlin, 1999; pp 302−310.
(4) Levy, S. B. *J. Antimicrob. Chemother.* **2002**, *49*, 25−30.
(5) D'Agata, E. M. C.; Jirjis, J.; Gouldin, C.; Tang, Y. W. *Am. J. Infect. Control* **2001**, *29*, 316−320.
(6) Bukharie, H. A.; Abdelhadi, M. S.; Saeed, I. A.; Rubaish, A. M.; Larbi, E. B. *Diagn. Microbiol. Infect. Dis.* **2001**, *40*, 1−4.
(7) Hancock, R. E. W. *Lancet* **1997**, *349*, 418−422.
(8) Gura, T. *Science* **2001**, *291*, 2068−2070.
(9) Li, P.; Chan, C.; He, B.; So, S. C.; Chung, Y. W.; Shang, Q.; Zhang, Y. D.; Zhang, Y. L. *Science* **2001**, *291*, 1783−1785.
(10) Hancock, R. E. W.; Diamond, G. *Trends Microbiol.* **2000**, *8*, 402−410.
(11) Barrett, G. C.; Elmore, D. T. *Amino Acids and Peptides*; Cambridge University Press: Cambridge, U.K., 1998; p 224.
(12) Kawulka, K.; Sprules, T.; McKay, R. T.; Mercier, P.; Diaper, C. M.; Zuber, P.; Vederas, J. C. *J. Am. Chem. Soc.* **2003**, *125*, 4726−4727.
(13) Wilson, K.-A.; Kalkum, M.; Ottesen, J.; Yuzenkova, J.; Chait, B. T.; Landick, R.; Muir, T.; Severinov, K.; Darst, S. A. *J. Am. Chem. Soc.* **2003**, *125*, 12475−12483.
(14) Barsby, T.; Kelly, M. T.; Gagne, S. M.; Andersen, R. J. *Org. Lett.* **2001**, *3*, 437−440.
(15) Bonnard, I.; Manzanares, I.; Rinehart, K. L. *J. Nat. Prod.* **2003**, *66*, 1466−1470.
(16) Ryan, M. P.; Jack, R. W.; Josten, M.; Sahl, H.-G.; Jung, G.; Ross, R. P.; Hill, C. *J. Biol. Chem.* **1999**, *274*, 37544−37550.
(17) Trishman, J. A.; Jensen, P. R.; Fenical, W. *Tetrahedron Lett.* **1994**, *35*, 5571−5574.
(18) Barsby, T.; Kelly, M. T.; Andersen, R. J. *J. Nat. Prod.* **2002**, *65*, 1447−1451.
(19) (a) Gerard, J.; Haden, P.; Kelly, M. T.; Andersen, R. J. *Tetrahedron Lett.* **1996**, *37*, 7201−7204. (b) Gerard, J. M.; Haden, P.; Kelly, M. T.; Andersen, R. J. *J. Nat. Prod.* **1999**, *62*, 80−85.
(20) Hill, R. L. In *Advances in Protein Chemistry*; Anfinsen, C. B., Anson, M. L., Edsall, J. T., Richards, F. M., Eds.; Academic Press: New York, 1965; Vol. 20, pp 37−107.

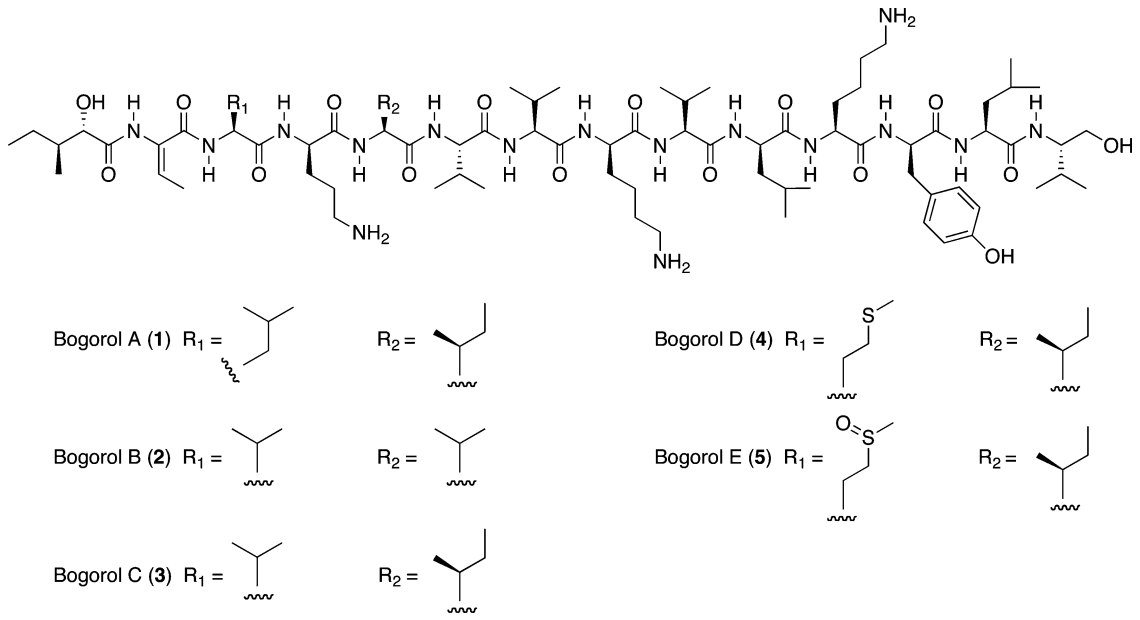


FIGURE 1. Bogorols A−E isolated from a marine isolate of *Brevibacillus laterosporus*.

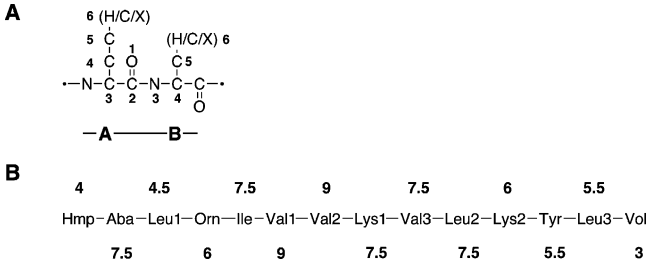


FIGURE 2. (A) Rule of six numbering system of a peptide bond. (B) Assignment of *residue average six* (*ras*) *numbers* in bogorol A.

hydrolyze at different rates. Newman evaluated the steric factors involved in acid-catalyzed esterification of carboxylic acids and proposed the empirical "*rule of six*", which stated that "in reactions involving addition to an unsaturated function, the greater number of atoms in the *six position*, the greater will be the steric effect".[21] The total number of atoms in the *six position* was referred to as the molecule's *six number*.[21] Whitfield reinterpreted the *rule of six* for the hydrolysis of dipeptides,[22] assigning *six number* values to dipeptides that correlated steric factors to the relative rates of dipeptide hydrolysis.

Using the generic peptide bond seen in Figure 2A, we can add the number of hydrogen, carbon, or heteroatoms that are at positions six to give a *six number* for a particular dipeptide, which correlates well with its hydrolysis rate characterized as rapid, moderate, or slow (a spread that covers approximately a factor of 60). The higher the *six number*, the slower the hydrolysis of the dipeptide. The contribution of an individual amino acid to the dipeptide's *six number* depends on its positioning at either site A or B of Figure 2A. However, assignment of configuration to an amino acid at a specific site in a peptide by analysis of hydrolysis rates requires an empirical value that combines the *six number* values for both dipeptides involving that residue. Averaging the *six numbers* for the two dipeptides involving each residue to give a *residue average six* (*ras*) *number* should allow prediction of hydrolytic liberation rates of the specific residues (Figure 2B) (see Supporting Information for further discussion on calculating *six numbers* and *ras numbers*).

The *ras numbers* of bogorol A's components predict that the first amino acid residues to appear should be Vol (3), Hmp (4), and Leu1 (4.5), followed by Leu3 (5.5), Tyr (5.5), Orn (6), and Lys2 (6) (Figure 2B). The slowest residues to appear should be Val1 (9) and Val2 (9), preceded slightly by Ile (7.5), Lys1 (7.5), Leu2 (7.5), and Val3 (7.5). *Ras number* analysis predicts that Lys2 (6) will be liberated more rapidly than Lys1 (7.5) and that the Leu residues should be liberated with the relative rates: Leu1 (4.5) > Leu3 (5.5) > Leu2 (7.5). To test the predicted amino acid liberation rates based on *ras number* analysis, a 6 N HCl (aq) solution of bogorol A (**1**) containing L-Ala as an internal standard was divided into six samples that were heated at 110 °C for differing time periods. After cooling and removal of solvents, the amino acids in the hydrolysates were converted to their PFPA-IPE derivatives and analyzed by chiral GC. Peak areas were measured relative to the peak area of the internal standard L-Ala (Figure 3).

As predicted, at the shortest reaction time (1.5 h) the hydrolysate contained mainly the residues Hmp (4), Vol (3), and Leu1 (4.5), which have the smallest *ras numbers*. In addition, only low levels of Val and Ile were observed throughout the hydrolysis and at $t = 48$ h. These observations validated the use of *ras numbers* as a proxy for a residue's

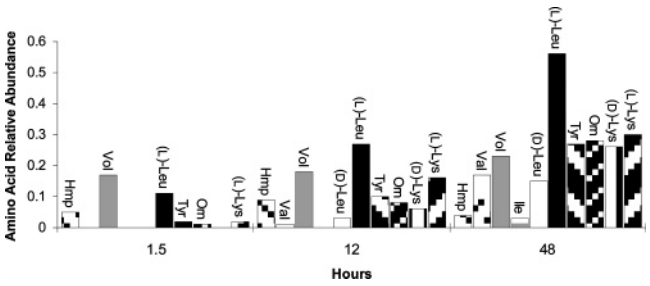


FIGURE 3. Relative abundance of constituents in hydrolysate of bogorol A (**1**) at three time points as determined by chiral GC.

(21) Newman, M. S. In *Steric Effects in Organic Chemistry*; Newman, M. S., Ed.; Wiley & Sons: New York, 1956; pp 201−248.
(22) Whitfield, R. E. *Science* **1963**, *142*, 577−579.

predilection for liberation during acid hydrolysis and provided a framework for site-specific configurational analysis of the Leu and Lys residues. At $t = 1.5$ h, only L-Leu is detected, leading to an assignment of the L configuration for Leu1, which has the smallest *ras number* of the three Leu residues. Since L-Leu was approximately twice as abundant as the other amino acids at $t = 48$ h, it was concluded that two of the three Leu residues in **1** are L-amino acids. The highly truncated levels of D-Leu throughout the hydrolysis led to the assignment of D-Leu at position Leu2 (7.5), which has the highest *ras number* of the three Leu positions. L-Lys is first detected at $t = 1.5$ h and is found to precede the appearance of D-Lys, which suggests that L-Lys is at position Lys2 (6), while D-Lys occupies Lys1 (7.5). Comparison of the relative ratios of D-Lys and L-Lys at times $t = 12$ h reveals that L-Lys is approximately twice as abundant, supporting the assignment of D-Lys at Lys1 and L-Lys at Lys2.

A classical partial peptide hydrolysis was undertaken to obtain the small peptide fragments needed to confirm the *ras number* kinetic analysis placement of the D and L Leu and Lys residues in bogorol A (**1**). Thus, a solution of bogorol A in 5 N HCl/MeCN (3:2) was partially hydrolyzed by being heated at 38 °C for 18 h. The small peptide fragments in the resulting hydrolysate were purified by reversed-phase HPLC and analyzed via ^{1}H NMR and ESI-MS. Among these fragments, a dipeptide-designated fragment **A** gave a $[M + H]^+$ ion at *m/z* 295.1 in the ESI-MS spectrum and its ^{1}H NMR and COSY spectra indicated the presence of Tyr and Leu residues (Supporting Information). On the basis of the sequence of bogorol A (**1**), fragment **A** had to be the dipeptide composed of Tyr and Leu3. A second peptide designated fragment **B** gave a $[M + H]^+$ ion at *m/z* 635.5 and a $[M + Na]^+$ ion at *m/z* 657.4 in the ESI-MS. The ^{1}H NMR and COSY spectra for fragment **B** (Supporting Information) indicated the presence of one Tyr, one Val, one Lys, and two Leu residues. It was apparent from the sequence of bogorol A that fragment **B** was a pentapeptide containing Val3-Leu2-Lys2-Tyr-Leu3. Fragment **A** containing Leu3 was hydrolyzed with 5 N HCl at 105 °C for 22 h, followed by Marfey's analysis of the hydrolysate, which showed that Leu3 was L. A similar analysis of fragment **B**, which contained Lys2, Leu2, and Leu3, revealed the presence of L-Lys and both L- and D-Leu, demonstrating that Lys2 was L and Leu2 was D. Since complete hydrolysis had shown that bogorol A contained two L-Leu, one D-Leu, one L-Lys, and one D-Lys, it was apparent that Leu1 was L and Lys1 was D, confirming the absolute configuration assignment made via the kinetic analysis.

Identification of the Bogorol MS Template. The structure of bogorol A (**1**) was originally elucidated by chiral GC analysis of derivatized amino acids obtained from an acid hydrolysate of the natural product in conjunction with interpretation of FAB-MS and 800 MHz NMR data obtained on its hexaacetyl derivative.[14] Using the MS fragmentation pattern of bogorol A (**1**) as a template, we were able to elucidate the structures of the co-occurring analogues on the basis of only MS data and standard GC amino acid analyses. The utilization of an electrospray ionization (ESI) instrument facilitated rapid acquisition of both MS and MS/MS (Figure 4) data using small amounts of bogorols (~10 μg).

The ESI-MS of bogorol A (**1**) (Figure 4A) showed a strong $[M + 1 + H]^+$ ion at *m/z* 1585.3, its sodium adduct $[M + 1 + Na]^+$ at *m/z* 1607.1, and the doubly charged species *m/2z* at 792.5 $[M + 1 + 2H]^{2+}$. Fragmentation proceeded from the Hmp-capped N-terminus, resulting in the loss of *m/z* 198

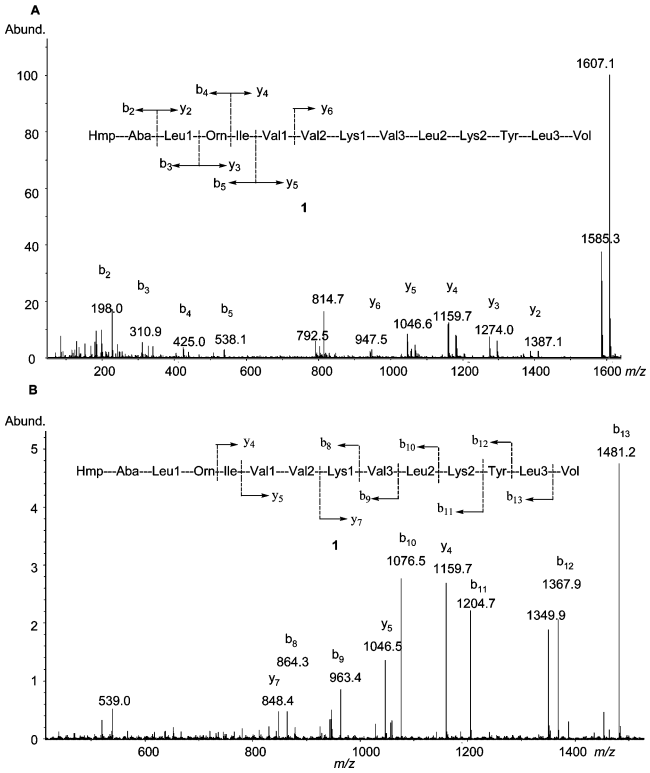
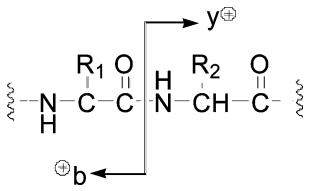


FIGURE 4. (A) ESI-MS of bogorol A (**1**). (B) ESI-MS/MS of the parent ion of bogorol A (**1**).

($\Delta_{M+1+H-y2}$, Hmp-Aba), followed by the consecutive loss of four amino acids: Leu1 (Δ_{y2-y3} 113), Orn (Δ_{y3-y4} 114), Ile (Δ_{y4-y5} 113), and Val1 (Δ_{y5-y6} 99). The MS/MS fragmentation of the bogorol A parent ion (Figure 4B) afforded the complementary fragmentation pattern that started with the loss of Vol to give b_{13}. The fragmentation continued with the loss of the next five consecutive amino acids: Leu3 ($\Delta_{b13-b12}$ 113), Tyr ($\Delta_{b12-b11}$ 163), Lys2 ($\Delta_{b11-b10}$ 128), Leu2 (Δ_{b10-b9} 113), and Val3 (Δ_{b9-b8} 99). Together, y_7 and b_8 positioned Lys1 ($\Delta_{(y7+b8)-1585}$ 128); also, y_7 and y_5 confirmed the positioning of the two consecutive valines, Val1-Val2 (Δ_{y5-y7} 198). Thus, the MS analysis confirmed the constitution of bogorol A (**1**) and provided a fragmentation template that could be used to rapidly elucidate the structure of the remaining bogorols (**2**−**5**).

Structure Elucidation of Bogorols B to E. Bogorol B (**2**) was isolated as an optically active white solid that gave a $[M + H]^+$ ion at *m/z* 1570.0710 in the HRESI-TOF mass spectrum, appropriate for the molecular formula $C_{79}H_{140}N_{16}O_{16}$, which suggested that **2** was a homologue of bogorol A (**1**). The ^{1}H NMR of **2**, like the ^{1}H NMR spectrum of **1**, showed the broad signals characteristic of a cationic peptide, with one notable difference being the presence of a new methine multiplet at δ 2.10, slightly deshielded from the valine β-methine multiplets at δ 1.95 in the spectrum of **1** (Supporting Information). Comparison of the ESI-MS and MS/MS data (Table 1, Supporting Information) for **2** with the data for **1** indicated that the mass difference resulted from substitution at the third amino acid position (AA3) where Δ_{b3-b2} is 99 Da for **2** and 113 Da for **1**, corresponding to a Val substitution for the L-Leu residue in **1**. Once the substitution at AA3 is accounted for, all the other MS fragment ions supported a shared constitution between **1** and **2**. Chiral GC analysis (Table 2) of the individual amino

TABLE 1. Summary of *m/z* Values for Daughter Ions in MS and MS/MS for Bogorols A−E (1−5)



ion	1	2	3	4	5
b_2	198.0	198.0	198.0	197.9	198.1
b_3	310.9	296.9	296.9	328.9	345.2
b_4	425.0	411.0	411.1	443.0	459.4
b_5	538.1	524.2	510.1	556.1	572.6
b_8	864.3	851.1	836.2	883.1	899.1
b_9	963.4	950.1	935.4	982.3	998.1
b_{10}	1076.5	1063.3	1048.6	1095.3	1111.2
b_{11}	1204.7	1191.3	1176.8	1223.3	1239.2
b_{12}	1367.9	1354.4	1339.9	1386.3	1402.4
b_{13}	1481.2	1467.4	1453.1	1499.5	1515.5
y_2	1387.1	1373.3	1359.3	1405.3	1421.1
y_3	1274.0	1274.1	1260.0	1274.0	1274.2
y_4	1159.7	1159.8	1145.9	1159.9	1160.2
y_5	1046.6	1046.8	1046.8	1046.6	1047.2
y_6	947.5	947.5	947.5	947.5	948.2
y_7	848.8	849.1	848.3	849.1	849.1

TABLE 2. Chiral GC Retention Times of Bogorol B−E (2−5) Hydrolysates[a]

standard	time (min)	2	3	4	5
R,S-Hmp/*S,R*-Hmp[b]	19.08				
S,S-Hmp/*R,R*-Hmp[b]	19.25	19.33	19.28	19.25	19.25
D-Val[b]	24.80				
L-Val[b]	25.15	25.10	25.10	25.09	25.08
S-Vol[b]	25.22	25.22	25.27	25.21	25.19
R-Vol[b]	25.32				
D-Ile	22.20				
L-Ile	22.62	22.61		22.52	22.52
D-Leu	23.80	23.72	23.68	23.72	23.69
L-Leu	24.61	24.52	24.50	24.55	24.51
D-Met[c]	33.10				
L-Met[c]	33.35			33.30	
L-Met(O)[c]	33.33				33.27
D-Tyr	37.00	36.97	36.92	36.97	36.92
L-Tyr	37.15				
D-Orn	37.82	37.77	37.71	37.79	37.74
L-Orn	37.99				
D-Lys	39.88	39.89	39.79	39.87	39.81
L-Lys	40.04	40.05	39.93	40.02	39.95

[a] All identifications were made by co-injections with standards. [b] A different temperature gradient was used with the other residues (see Experimental in Supporting Information). [c] Under the hydrolysis conditions, Met is oxidized to Met(O).[23]

acids in the hydrolysate of **2** revealed that bogorol B contained the same residues present in **1**. In particular, no signal for D-Val was present, which enabled the mass difference to be assigned to an L-Val for L-Leu substitution at the AA3 position of **1**.

This substitution of an L-Val in **2** for the L-Leu at AA3 in **1** is consistent with a putative nonribosomal biosynthesis for the bogorols (**1**−**5**). Nonribosomal peptide synthetases are megaenzymes organized as modules, each containing a sequence of linked catalytic domains that are required for the incorporation of individual amino acid residues in the growing peptide chain.[24] The adenylation domain that is responsible for the selection and attachment of the amino acids to the megaenzyme synthetase can show relaxed substrate specificity, resulting in families of peptides with defined sequence positions where amino acid variations occur. Typically, the epimerization of an amino acid occurs further along the module, after its attachment as a thioester on the peptidyl carrier protein domain. If epimerization is required within a particular module, then the module will contain an epimerization domain. Thus, as is observed with the substitution of L-Val for L-Leu in going from **1** to **2**, the position-specific configuration within a family of nonribosomal peptides can be viewed as defined, despite the inherent possibility of amino acid substitutions.

During the GC analyses of acid hydrolysates from **2**−**5**, the Hmp residue was detected as its *O*-pentafluoropropionyl isopropyl ester, for which chiral resolution was not possible. It was shown that the Hmp acid obtained from **2** belonged to the same pair of diastereomers as the Hmp acid of **1**, and by the previous nonribosomal peptide biogenetic arguments, they were assumed to be equivalent. In addition, the presence of the diagnostic olefinic methine and methyl (H_B-2 and H_B-3) signals of the Aba residue observed in the ^{1}H NMR spectrum of **2** confirmed its existence, which had been suggested by the b_2 fragment in the ESI-MS. The ^{1}H chemical shift of the Aba residue's olefinic methyl group (H_B-3) is documented to be particularly sensitive to the olefin's configuration, with a typical difference of 0.31 ± 0.03 ppm between the *E* and *Z* configurations.[25] Given that the chemical shift of H_B-3 is conserved at 1.76 ± 0.02 ppm within the bogorol family (**1**−**5**) (see Table 1 of the Supporting Information), the olefins in **2**−**5** were all assigned as *E*, which was previously determined for **1** through a NOESY experiment. Thus, by working with the structural and configurational template established for **1**, the structure elucidation of **2** was completed.

Bogorol C (**3**) was isolated as an optically active white solid that gave a $[M + H]^+$ ion at *m/z* 1556.0565 in the HRESI-TOFMS appropriate for the molecular formula $C_{78}H_{138}N_{16}O_{16}$, which indicated that **3** was a homologue of **1** and **2**. The ^{1}H NMR spectrum of bogorol C (**3**) (Supporting Information) was nearly identical to the spectrum of bogorol B (**2**). However, the ESI-MS and MS/MS fragmentation (Table 1, Supporting Information) isolated the mass difference as occurring from the substitution of Val (**3**, Δ_{b3-b2} 99; Δ_{b5-b4} 99) for both the Leu at the AA3 (**1**, Δ_{b3-b2} 113) and the Ile at the AA5 (**1**, Δ_{b5-b4} 113) positions in **1**. Chiral GC analysis (Table 2) of the acid hydrolysate confirmed the lack of an Ile or D-Val residue and showed that the remaining amino acid composition of **3** was consistent with the proposed structure.

Bogorol D (**4**) was isolated as an optically active white solid that gave a $[M + H]^+$ ion at *m/z* 1602.0437 in the HRESI-TOF mass spectrum appropriate for the molecular formula $C_{79}H_{140}N_{16}O_{16}S$. The molecular formula indicated that the loss of CH_2, coupled with the addition of a sulfur atom, led to the increase of 18 Da over the mass of **1**. Inspection of the ^{1}H NMR spectrum of **4** (Supporting Information) revealed the presence of a sharp methyl singlet at δ 2.05, an appropriate chemical shift for a methyl sulfide. The ESI-MS and MS/MS (Table 1, Supporting Information) fragmentation and the chiral GC analysis (Table 2) of the acid hydrolysate identified a Met (**4**, Δ_{b3-b2} 131)

(23) Blackburn, S. In *Amino Acid Determination: Methods and Techniques*, 2nd ed.; Blackburn, S., Ed.; Marcel Dekker: New York, 1978; pp 7−38.

(24) Schwarzer, D.; Marahiel, M. A. *Naturwissenschaften* **2001**, *88*, 93−101.

(25) Srinivasan, A.; Stephenson, R. W.; Olsen, R. K. *J. Org. Chem.* **1977**, *42*, 2256−2260.

TABLE 3. Minimum Inhibitory Concentration (μg/mL) of Bogorols A−E (1−5) As Determined by Agar Dilution Using Mueller-Hinton Media

compd	MRSA	VRE	*E. coli*
1	2.5	9.0	37
2	2.5	9.0	37
3	2.5	75	75
4	2.5	18	75
5	4.5	18	>200

substitution for L-Leu (**1**, Δ_{b3-b2} 113) at AA3. The GC and MS analyses revealed that the remaining structural elements of **4** were identical to those of **1** and confirmed that the Met-3 residue had the expected L configuration.

Bogorol E (**5**) was isolated as an optically active white solid that gave a $[M + H]^+$ ion at *m*/*z* 1618.0405 in the HRESI-TOF mass spectrum, appropriate for the molecular formula $C_{79}H_{140}N_{16}O_{17}S$, which suggested that **5** was the oxidation product of **4**. Consistent with this suggestion, the ^{1}H NMR spectrum of **5** (see Supporting Information) contained a methyl singlet at δ 2.50, appropriate for a methyl sulfoxide. Further inspection of the ESI-MS and MS/MS fragmentation (Table 1, Supporting Information), coupled with chiral GC analysis of the component amino acids (Table 2), revealed that the mass difference of **5**, relative to that of **1**, resulted from a L-Met(O) (**5**, Δ_{b3-b2} 147) substitution for L-Leu (**1**, Δ_{b3-b2} 113) at AA3.

Typically, methionine sulfoxide-containing peptides are isolated as a mixture of diastereomers as a result of the presence of both *R* and *S* methyl sulfoxides, which is indicated by a doubling of the methyl sulfoxide signal in both the ^{1}H and ^{13}C NMR spectra.[26,27] With bogorol E (**5**), no doubling of signals was observed in the ^{1}H NMR spectrum and limited material precluded acquiring a ^{13}C NMR spectrum. During the HPLC purification a closely eluting fraction, of equivalent molecular mass, was found that could not be sufficiently purified for rigorous identification. Together with the lack of the doubling of the Met(O) methyl ^{1}H NMR signal, this observation suggests that the *R* and *S* sulfoxides were resolved. However, the identity of which diastereomer is present in **5** was undetermined.

Biological Activity. Bogorols A−E (**1**−**5**) were tested for antibacterial activity against a panel of human pathogens including MRSA, VRE, and *E. coli* (Table 3). The bogorols were all inactive (MICs > 200 μg/mL) against *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, and *Candida albicans* but displayed good activity versus MRSA, which falls within the 1−8 μg/mL range found for other cationic peptide antibiotics.[7] Intriguingly, simple amino acid substitutions to the bogorol template resulted in some pronounced differences in biological data. In particular, the change from **2** to **3** is a simple Val for Ile substitution, yet **2** is 8 times more active against VRE.

Conclusions

The structure elucidation of bogorol A (**1**) has been used as a venue to both illustrate and validate an empirical method based on *rule of six* (*ras*) *numbers* to position D and L Leu and Lys residues in the linear peptide sequence. This method, which offers advantages over traditional degradation techniques because it relies on small amounts of sample, has the potential to be generally applicable.

The bogorols contain a number of features that are typical of nonribosomal peptides. These include the reduction of the C-terminal residue to give valinol (Vol), transformation of the N-terminal Ile to 2-hydroxy-3-methylpentanoic (Hmp) acid, incorporation of four D amino acids, and the presence of a dehydroamino acid (Aba). As a consequence of the C- and N-terminal modifications, all of the potentially charged residues in the bogorols reside in the interior of the linear peptide chain. The bogorols appear to be the first known linear cationic peptide antibiotics with both C-terminal aminol and N-terminal α-hydroxy acid modifications. Structural variations in the bogorol family result from amino acid substitutions only at the AA3 and AA5 positions in the sequence, suggesting that the putative nonribosomal peptide synthetase involved in making these linear peptides has a reduced substrate specificity in the adenylation domain for these two positions.

The bogorols showed selective and relatively potent activity against MRSA and VRE, as well as moderate activity versus *E. coli*. Since they represent a new cationic peptide antibiotic template, they are attractive leads for a structure−activity relationship study aimed at optimizing their antibacterial potential.

Experimental Section

Isolation of the Bogorols. Lyophilized *B. laterosporus* PNG-276 cells (21.5 g) were immersed in MeOH and extracted three times over a period of 6 days. The MeOH extracts were combined, filtered, and concentrated in vacuo to give a brown/gray tar. The tar was taken up in 200 mL of H_2O/MeOH (10:1) and partitioned with 100% EtOAc (3 × 100 mL). The combined EtOAc extracts were reduced in vacuo to give a crystalline solid (6.5 g). In two portions, the residue was subjected to size exclusion chromatography on a Sephadex LH-20 (100% MeOH) column to give 500 mg of a fast eluting, ninhydrin positive fraction. This fraction was loaded onto a reversed-phase 10 g Sep-Pak previously equilibrated with 100% H_2O, by dissolving the sample in a minimum of MeOH. Upon addition of the MeOH solution to the top of the column, an equivalent volume of H_2O was added, precipitating the active component. The column was flushed first with 50 mL of 100% H_2O, which was subsequently discarded, followed by 100 mL of H_2O/MeCN (6:4) with 0.2% TFA. The 6:4 H_2O/MeCN eluate gave 90 mg of a mixture of large molecular weight peptides (1556−1618 Da) which were responsible for the observed anti-*E. coli* biological activity. The crude peptides were further separated into nine fractions by reversed-phase HPLC using a Dynamax-60 C18 column, eluting with H_2O/MeCN (6:4) and 0.2% TFA. Repeated HPLC recycling using an Inertsil C18 column resulted in the isolation of bogorols A (**1**, 3.2 mg), B (**2**, 4.1 mg), C (**3**, 1.5 mg), D (**4**, 4.4 mg), and E (**5**, 2.5 mg).

Bogorol A (1): isolated as a white solid that was deemed to be >95% pure by ^{1}H NMR spectroscopy; $[\alpha]^{25}_D$ −38.2° (MeOH); for ESI-QIT-MS and MS/MS data see Table 1 and Supporting Information; for GC data see Table 2; HRESIMS $[M + H]^+$ *m*/*z* 1584.0875 ($C_{80}H_{143}N_{16}O_{16}$, calcd 1584.0868; ΔM = +0.4 ppm).

Bogorol B (2): isolated as a white solid that was deemed to be >95% pure by ^{1}H NMR spectroscopy; $[\alpha]^{25}_D$ −50.3° (MeOH); for ESI-QIT-MS and MS/MS data see Table 1 and Supporting Information; for GC data see Table 2; HRESIMS $[M + H]^+$ *m*/*z* 1570.0710 ($C_{79}H_{141}N_{16}O_{16}$, calcd 1570.0711; ΔM = −0.1 ppm).

Bogorol C (3): isolated as a white solid that was deemed to be >95% pure by ^{1}H NMR spectroscopy; $[\alpha]^{25}_D$ −64.7° (MeOH); for ESI-QIT-MS and MS/MS data see Table 1 and Supporting Information; for GC data see Table 2; HRESIMS $[M + H]^+$ *m*/*z* 1556.0565 ($C_{78}H_{139}N_{16}O_{16}$, calcd 1556.0555; ΔM = +0.6 ppm).

(26) Ogino, J.; Moore, R. E.; Patterson, G. M. L.; Smith, C. D. *J. Nat. Prod.* **1996**, *59*, 581−586.
(27) Harrigan, G. G.; Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Nagle, D. G.; Paul, V. J. *J. Nat. Prod.* **1999**, *62*, 655−658.

Bogorol D (4): isolated as a white solid that was deemed to be >95% pure by ^{1}H NMR spectroscopy; $[\alpha]^{25}_D$ −43.5° (MeOH); for ESI-QIT-MS and MS/MS data see Table 1 and Supporting Information; for GC data see Table 2; HRESIMS $[M + H]^+$ *m*/*z* 1602.0437 ($C_{79}H_{141}N_{16}O_{16}S$, calcd 1602.0432; $\Delta M = +0.3$ ppm).

Bogorol E (5): isolated as a white solid that was deemed to be >95% by ^{1}H NMR spectroscopy; $[\alpha]^{25}_D$ −17.2° (MeOH); for ESI-QIT-MS and MS/MS data see Table 1 and Supporting Information material; for GC data see Table 2; HRESIMS $[M + H]^+$ *m*/*z* 1618.0405 ($C_{79}H_{141}N_{16}O_{17}S$, calcd 1618.0381; $\Delta M = +1.5$ ppm).

Total Acid Hydrolysis and GC Analysis. In a screw-top vial, bogorols (0.5 mg) were dissolved in 6 N HCl (1 mL) and heated at 110 °C for 72 h. The HCl was removed under a stream of N_2 gas. A stock solution of *i*-propyl acetate was created by slowly adding 1.25 mL of acetyl chloride to 5 mL of *i*-propanol at 0 °C. *i*-Propyl acetate (250 μL) was added to the reaction vial and heated to 110 °C for a further 45 min, followed by solvent removal under a stream of N_2 gas. Dichloromethane (250 μL) and pentafluoropropionyl anhydride (100 μL) were added to the reaction vial and heated to 110 °C for 15 min. Excess reagent was removed under N_2 gas, and the sample was redissolved in dichloromethane (200 μL). Standards were prepared in the same fashion using optically pure L amino acids and their racemic mixtures. The amino acid standards and the hydrolysates were analyzed on a 25-m chiralsil-Val Heliflex column with FID detection using the following conditions: He carrier; initial oven temp 60 °C until initial time 4 min; program rate 3 °C/min to oven temp 130 °C; program rate 10 °C/min to oven temp 190 °C (37.5 min.); isothermal until final time 47.5 min. For the analysis of the valine, valinol, and the 2-hydroxy-3-methylpentanoic acid derivatives, the following temperature gradient was used: initial oven temp 60 °C; initial time, 15 min; program rate 4 °C/min to oven temp 80 °C; program rate 10 °C/min to oven temp 190 °C; isothermal until final time of 47.5 min.

Partial Hydrolysis Experiment. To 1.5 mg of bogorol A (**1**) (0.95 μmol) was added 1.5 mL of 6 N HCl containing 3.84 μmol L-Ala. The solution was mixed and then divided into the six 0.25-mL samples in screw-top vials. The vials were sealed and placed in a sand bath heating mantle at 105 °C ($t = 0$); one sample was removed at each of $t = 1.5$, 3, 6, 12, 24, and 48 h and subjected to the procedure outlined above. The same column conditions were used as in the earlier section.

Isolation of Partial Hydrolysis Fragments A and B. Bogorol A (**1**) (20 mg) was hydrolyzed in a mixed solution of 6 N HCl/MeCN (5:2) (1.4 mL) at 38 °C for 18 h. After neutralization with 5 N NaOH, the reaction mixture was fractionated by reversed-phase HPLC on CSC-Inertsil 150A/ODS2 (ϕ 9.4 × 250 mm) using a linear gradient elution from 10 to 70% aqueous MeCN containing 0.2% TFA. The fraction containing fragment **A** was further purified by reversed-phase HPLC on CSC-Inertsil 150A/ODS2 (ϕ 9.4 × 250 mm) eluted with 31% aqueous MeCN containing 0.2% TFA to afford pure fragment **A** (0.1 mg). Similarly, the fraction containing fragment **B** was purified by ODS HPLC eluting with 25% aqueous MeCN containing 0.2% TFA to yield pure fragment **B** (0.5 mg).

Fragment A: colorless solid; ^{1}H NMR (CD_3OD, 600 MHz) δ 7.06 (2H, d, $J = 8.3$ Hz), 6.76 (2H, d, $J = 8.3$ Hz), 4.20 (1H, brd), 4.01 (1H, t, $J = 8.0$ Hz), 3.00 (2H, m), 1.50 (2H, m), 1.17 (1H, m), 0.85 (3H, d, $J = 6.4$ Hz), 0.80 (3H, d, $J = 5.8$ Hz); LR-ESIMS *m*/*z* 295.1 $[M + H]^+$.

Fragment B: colorless solid; ^{1}H NMR (CD_3OD, 600 MHz) δ 7.05 (2H, d, $J = 8.6$ Hz), 6.70 (2H, d, $J = 8.6$ Hz), 4.67 (1H, t, $J = 7.5$ Hz), 4.38 (1H, dd, $J = 5.8$, 9.1 Hz), 4.31 (1H, m), 4.30 (1H, m), 3.67 (1H, d, $J = 5.8$ Hz), 2.95 (1H, dd, $J = 8.3$, 13.7 Hz), 2.88 (2H, m), 2.84 (1H, dd, $J = 7.2$, 13.7 Hz), 2.20 (1H, hext, $J = 6.8$ Hz), 1.81 (1H, m), 1.66−1.57 (8H), 1.50 (1H, m), 1.31 (1H, m), 1.22 (1H, m), 1.07 (3H, d, $J = 6.8$ Hz), 1.05 (3H, d, $J = 6.8$ Hz), 0.99 (3H, d, $J = 6.4$ Hz), 0.95 (3H, d, $J = 6.4$ Hz), 0.87 (3H, d, $J = 6.5$ Hz), 0.82 (3H, d, $J = 6.5$ Hz); LR-ESIMS *m*/*z* 635.5 $[M + H]^+$ and 657.4 $[M + Na]^+$.

Determination of the Absolute Configuration of Amino Acids in Fragment A.[28] Fragment **A** (50 μg) was hydrolyzed with 5 N HCl (200 μL) at 105 °C for 22 h. The reaction solution was lyophilized to remove the solvent. To the residue, 30 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (1 mg/mL) and 60 μL of 1 N $NaHCO_3$ were added. The reaction solution was kept at 65 °C for 10 min. To the reaction mixture, 30 μL of 0.2 N HCl and 60 μL of 50% aqueous MeCN containing 0.1% TFA were added, and the mixture was analyzed by reversed-phase HPLC on Alltech Econosil C18 (ϕ 4.6 × 250 mm) using isochratic elution with 26% aqueous MeCN containing 0.05% TFA over 5 min followed by a linear elution gradient from 26 to 56% aqueous MeCN containing 0.05% TFA over 55 min at a flow rate of 1 mL/min. All identifications of the amino acids were made by co-injections with standards. Retention times (min): L-Tyr (24.0), D-Tyr (26.0), L-Leu (34.8), D-Leu (40.2).

Determination of the Absolute Configuration of the Amino Acids of Fragment B.[28] Fragment **B** (200 μg) was hydrolyzed with 5 N HCl (300 μL) at 105 °C for 19 h. The reaction solution was lyophilized to remove the solvent. To the residue, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (1 mg/ mL) and 100 μL of 1 N $NaHCO_3$ were added. The reaction solution was kept at 65 °C for 10 min. To the reaction mixture, 50 μL of 0.2 N HCl and 100 μL of 50% aqueous MeCN containing 0.1% TFA were added, and the mixture was analyzed by reversed-phase HPLC on Alltech Econosil C18 (ϕ 4.6 × 250 mm) using either the elution program of an isochratic elution with 18% aqueous MeCN containing 0.2% TFA over 5 min and a linear elution from 18 to 50% aqueous MeCN containing 0.2% TFA at a flow rate of 1 mL/min for Lys or that of an isochratic elution with 26% aqueous MeCN containing 0.05% TFA over 5 min and a linear gradient elution from 26 to 58% aqueous MeCN containing 0.05% TFA over 55 min at a flow rate of 1 mL/min for Tyr, Val, and Leu. All identifications of the amino acids were made by co-injections with standards. Retention times (min): D-Lys (30.6 and 33.6), L-Lys (32.4 and 33.6), l-Tyr (24.6), D-Tyr (29.4), L-Val (36.0), D-Val (45.0), L-Leu (45.6), D-Leu (50.4).

Acknowledgment. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada to R.J.A. and T.B. We thank Helen Wright for culturing *B. laterosporus*.

Supporting Information Available: Copies of the ^{1}H NMR of **1**−**5** and fragments A and B; ESI-MS and MS/MS spectra of **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO060667P

(28) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.