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Viequeamide A, a Cytotoxic Member of the Kulolide Superfamily of Cyclic Depsipeptides from a Marine Button Cyanobacterium

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Abstract

The viequeamides, a family of 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya) containing cyclic depsipeptides, were isolated from a shallow subtidal collection of a ‘button’ cyanobacterium (*Rivularia* sp.) from near the island of Vieques, Puerto Rico. Planar structures of the two major compounds, viequeamide A (**1**) and viequeamide B (**2**), were elucidated by 2D-NMR spectroscopy and mass spectrometry, whereas absolute configurations were determined by traditional hydrolysis, derivative formation, and chromatography in comparison with standards. In addition, a series of related minor metabolites, viequeamide C–F (**3–6**), were characterized by high resolution mass spectroscopic (HRMS) fragmentation methods. Viequeamide A was found to be highly toxic to H460 human lung cancer cells (IC₅₀ = 60 ± 10 nM), whereas the mixture of B–F was inactive. From a broader perspective, the viequeamides help to define a “superfamily” of related cyanobacterial natural products, the first of which to be discovered was ‘kulolide’. Within the kulolide superfamily, a wide variation in biological properties is observed, and the reported producing strains are also highly divergent, giving rise to several intriguing questions about structure-activity relationships and the evolutionary origins of this metabolite class.

Marine cyanobacteria are increasingly being recognized as important sources of biologically active secondary metabolites.^{1–3} While numerous natural products have been reported from marine invertebrates, subsequent work has shown that in many cases, the true sources of compounds of interest are bacteria.^{4,5} Efforts by many research groups to directly focus on marine bacteria and cyanobacteria, have been rewarded by the discovery of an impressive number of highly cytotoxic compounds, such as curacin A,⁶ largazole,⁷ the carmaphycins,⁸ and salinosporamide A.⁹ More broadly, the diversity of marine natural products discovered so far, reveals an untapped wealth of structural diversity and activities well worth exploring.

Within marine natural products, depsipeptides produced by a hybrid pathway of nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), have been extensively reported. These biosynthetic pathways, as well as specific modifications, such as *N*-methylation, are characteristic of several groups of filamentous marine cyanobacteria.^{1,2} For example since the isolation of kulolide in 1993 by Scheuer and coworkers from the cephalaspidean mollusk *Philineopsis speciosa*,¹⁰ a variety of structurally similar compounds

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have been isolated from diverse cyanobacteria. These related compounds contain either a 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya) or 3-hydroxy-2-methyl-7-octynoic acid (Hmoya) moiety within an overall cyclic depsipeptide framework, and suggests the existence of a 'kulolide superfamily' within the natural products of these filamentous prokaryotes.^{10–21} The striking structural similarities within this large metabolite class, as well as their diverse reported metabolic sources, suggests that the kulolide superfamily may have an ancient evolutionary origin within the cyanobacteria.

In the current study, an extraction of a marine cyanobacterium collected from the Puerto Rican island of Vieques, showed a ¹H NMR spectrum with peaks characteristic of a depsipeptide. Purification of this material afforded a major compound, viequeamide A (**1**), that was found to be highly cytotoxic to the H460 cell line. The structure of this compound and a related minor metabolite were determined by 2D-NMR and mass spectroscopic (MS) methods, while the planar structures of a series of very minor metabolites were determined by MS fragmentation methods. Absolute configuration of the two more major compounds, viequeamide A (**1**) and B (**2**), were determined by hydrolysis to fragments which were converted into various derivatives and analyzed by chromatography in comparison with standards.

RESULTS AND DISCUSSION

A 'button' cyanobacterium was collected from the Puerto Rican island of Vieques, preserved in 1:1 isopropyl alcohol—seawater, and later extracted for its lipid soluble metabolites. The extract was subjected to a preliminary Vacuum Liquid Chromatography (VLC) fractionation scheme. High Performance Liquid Chromatography (HPLC) purification of the relatively polar ethyl acetate eluted fraction afforded a single major compound, viequeamide A (**1**, 13.6 mg). Additionally, viequeamides B–D (**2–4**) and two unresolved compounds viequeamides E and F (**5** and **6**), were obtained as inseparable mixtures, as described below.

HRMS analysis of viequeamide A (**1**) showed a compound with an $[M + Na]^+$ m/z of 826.4940, providing the molecular formula C₄₂H₆₉N₅O₁₀ (11 degrees of unsaturation). The ¹³C NMR spectrum showed seven carbonyl peaks at δ 168 – 174, thus leaving unaccounted four additional double bonds or rings in the molecule (Table 1).

Total Correlation Spectroscopy (TOCSY) experiments revealed eight proton spin systems. Four of these were for easily recognized amino acid residues, namely threonine, proline (accounting for one more degree of unsaturation), and two *N*-methyl valine residues. Two of the remaining TOCSY-defined spin systems were connected by Correlation Spectroscopy (COSY), suggesting constrained rotation might be preventing strong TOCSY correlations within this valine residue. Furthermore, a combination of Heteronuclear Multiple Bond Correlation (HMBC) and Heteronuclear Single Quantum Correlation (HSQC) experiments confirmed that these two spin systems belonged to a valine residue wherein a very small ¹H-¹H coupling was present between the α and β -methine protons ($J = 2.3$). A third unassigned and very weak TOCSY spin system was better revealed by COSY correlations, and involved a doublet proton at δ_H 4.82 that showed a small ¹H-¹H coupling constant ($J = 2.0$) to an adjacent methine at δ_H 1.72 (m). The HSQC spectrum indicated that the methine proton at δ_H 4.82 was bonded to a carbon atom at δ_C 74.5, strongly suggesting that this carbon had an attached oxygen atom. From the COSY, HMBC, and HSQC spectra the δ_H 4.82 doublet was shown to be the α methine proton of an Hmpa residue, the hydroxy acid analog of isoleucine. The final TOCSY spin system was characteristic of a PKS derived residue, as it contained a chain of adjacent methylenes (δ_C 27.9, 24.8, 18.1) and an oxygen-bearing methine (δ_C 77.4). HMBC correlations from two singlet methyl groups (δ_H 1.18 and

1.36) to a quaternary carbon (δ_C 46.7), the final unaccounted for carbonyl carbon at δ_C 174.7, and the oxygen bearing carbon atom at δ_C 77.4, defined an α,α -dimethyl- β -hydroxy-carbonyl system. Additionally, the distal methylene group of the methylene chain (δ_H 2.19–2.32) displayed HMBC correlations to carbons at δ_C 84.2 and 68.9, revealing that this residue terminated with an alkyne group, and therefore accounted for the 9th and 10th degrees of unsaturation present in viequeamide A (**1**). With consideration of the molecular formula, this latter residue was defined as a derivative of 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya).

The sequence of residues in **1** was elucidated by HMBC and Rotating-frame Overhauser Effect Spectroscopy (ROESY) experiments. Beginning with the Dhoya residue, both the valine α -methine proton and nitrogen bearing proton showed HMBC correlations to the Dhoya carbonyl carbon at δ_C 174.7, showing that an amide connected these two residues. In turn, the methyl group of *N*-methyl valine-1 showed HMBC correlations to the valine carbonyl carbon at δ_C 172.8, showing an *N*-methyl amide group connected these two residues. The α -methine proton in Hmpa at δ_H 4.82 showed HMBC correlation to the carbonyl carbon of *N*-methyl valine-1 at δ_C 170.3, revealing that there was an ester linkage between these two residues. It was noted that the α -methine proton of Hmpa at δ_H 4.82 showed no HMBC correlations across its carbonyl carbon to the next residue in the sequence. By ROESY, however, a correlation was observed between the α -methine proton of Hmpa and the δ -protons of the proline residue. We have previously observed in this class of compound that HMBC correlations to a proline residue are not easily observed;²¹ however, this ROESY correlation provided the amide connection of Hmpa to proline. To the other side of the proline, the *N*-methyl protons of *N*-methyl valine-2 showed strong HMBC correlation to the proline carbonyl carbon at δ_C 172.1, indicating the linkage of these two residues through an *N*-methyl amide. Both the amide nitrogen proton and the α -methine proton of threonine showed HMBC correlations to the carbonyl carbon of *N*-methyl valine-2 at δ_C 168.2, showing these residues were linked by an amide bond. Completing the residue sequence, and accounting for the final 11th degree of unsaturation, the oxygen-bearing β -methine proton of the Dhoya residue showed HMBC correlation to the carbonyl of threonine at δ_C 169.2, indicating the linkage of these two residues through an ester, thus completing an overall macrocyclic structure for viequeamide A (**1**). Summarizing, a combination of HMBC and ROESY correlation data allowed full definition of the residue sequence of **1** as cyclo-[Dhoya–Val–*N*-Me-Val-1–Hmpa–Pro–*N*-Me-Val-2–Thr] (Table 1, Figure 1).

The configurations of the amino acids in viequeamide A were all determined to be L by hydrolysis and Marfey's analysis^{22a–b} whereas the configuration of the Hmpa residue was established to be 2*R*,3*S* by chiral gas-column mass spectrometry (GCMS) in comparison to synthetic standards.^{21,23} The configuration of the Dhoya residue was determined by hydrogenation of **1** using hydrogen gas over palladium on carbon and then hydrolysis to release the 2,2-dimethyl-3-hydroxyoctanoic acid (Dhooa) unit; this was compared by GCMS to standards previously prepared,²⁴ thus establishing the *S* configuration and complete stereostructure of viequeamide A (**1**).

Several analogs of viequeamide A (**1**), namely viequeamides B–F (**2–6**), were also isolated from this extract. Unfortunately, these latter derivatives were inseparable by HPLC possibly because of the presence of equilibrating rotomers of each resulting from multiple *N*-methyl amide functional groups as well as their very similar structures. While the planar structure of the major compound in the mixture, viequeamide B, could be determined by 2D-NMR as described below, the minor constituents could only be characterized by the use of HRMS fragmentation experiments.

By inspection of the ^1H and ^{13}C NMR spectra for viequeamides B (**2**) in comparison with that of viequeamide A (**1**), these metabolites were clearly very closely related with both possessing two *N*-methyl amide peaks, similar doublet methyl groups indicative of valine, leucine, or isoleucine residues, and the alkyl methylene and singlet methyl protons indicative of a Dhoya residue. The most striking difference between the two was the presence of aryl protons in viequeamide B (**2**) which were absent in A (**1**). By the interplay of TOCSY, COSY, HSQC, and HMBC experiments, the residues of viequeamide B (**2**) were elucidated as Dhoya, proline, valine, *N*-methyl valine, and two residues not observed in viequeamide A, phenyl lactic acid (Pla) and *N*-methyl alanine. The sequence of residues of **2** was deduced from ROESY and HMBC correlations as cyclo-[Dhoya-Val-1-*N*-Me-Val-Pla-Pro-*N*-Me-Ala-Val-2] (Table 2, Figure 1).

Stereoanalysis of this viequeamide B dominated mixture of compounds **2–6**, was undertaken with the assumption that the minor natural products (**3–6**) would contribute negligibly to the analysis (see analysis of molecular ions below for relative concentrations). Thus, the mixture was first hydrogenated to reduce the unstable acetylene functional group of the Dhoya residue and then subjected to microwave-assisted acid hydrolysis. A portion of the hydrolysate was converted with 1-fluoro-2,4-dinitrophenyl-5-L-valine amide (L-FDVA) to Marfey's-type derivatives and compared by LCMS with authentic amino acids similarly derivatized, revealing only L residues (L-*N*-Me-Ala, L-Val, L-*N*-Me-Val, and L-Pro). A second portion of the hydrolysate was converted to methyl esters with CH_2N_2 and then analyzed under two different GCMS run conditions to identify (*S*)-2,2-dimethyl-3-hydroxyoctanoic acid (*S*-Dhooa, and by inference *S*-Dhoya) and *S*-phenyl lactic acid, thus completing the stereostructure of viequeamide B (**2**).

Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS) of the mixture of compounds dominated by **2** gave the major molecular ions at m/z 830.4666, 832.4833, 834.4988, and 844.4833, present in the approximate ratio of 25:5:1:1, respectively. These corresponded to the $[\text{M} + \text{Na}]^+$ ions for compounds with the molecular formulas of $\text{C}_{44}\text{H}_{65}\text{N}_5\text{O}_9$, $\text{C}_{44}\text{H}_{67}\text{N}_5\text{O}_9$, and $\text{C}_{44}\text{H}_{69}\text{N}_5\text{O}_9$, for viequeamides B–D, and $\text{C}_{45}\text{H}_{67}\text{N}_5\text{O}_9$ for viequeamides E/F which are isobaric. The molecular formulas of compounds **3** and **4**, $\text{C}_{44}\text{H}_{67}\text{N}_5\text{O}_9$ and $\text{C}_{44}\text{H}_{69}\text{N}_5\text{O}_9$, suggested that they might be related to viequeamide B (**2**) by varying levels of saturation of the alkyne functional group in the Dhoya residue. These hypotheses were confirmed by MS^2 fragmentation experiments which showed that viequeamide C (**3**) had a fragmentation pattern consistent with the alkene analog 2,2-dimethyl-3-hydroxyoctenoic acid (Dhoea), in viequeamide B, whereas viequeamide D (**4**) agreed well with the corresponding alkane analog, 2,2-dimethyl-3-hydroxyoctanoic acid (Dhooa).

In the metabolites with heaviest m/z value, **5** and **6**, the additional 14 amu mass (relative to **2**) could be explained by elongation of the PKS chain by one carbon, methylation of an amide residue, or the exchange of a valine residue in viequeamide B (**2**) by either leucine or isoleucine. Surprisingly fragmentation of the parent ion of 822 m/z showed two overlapping fragmentation patterns, revealing that this peak was composed of a mixture of two isobaric compounds possessing the same molecular formula. Using the nonribosomal peptide sequencing tool previously developed by our laboratories,²⁵ the structures of viequeamides E (**5**) and F (**6**) were found to be analogous to viequeamide B (**2**), with the addition of 14 mass units located alternately on Val-1 or Val-2 to either side of the Dhoya residue. In viequeamide E (**5**) Val-1 was modified whereas, in viequeamide F (**6**) Val-2 that was modified (see Figure 2). Unfortunately fragmentation experiments could not resolve the identity of the residues in these two compounds as either leucine, isoleucine, or *N*-methyl valine as all three have identical molecular formulas which would match the observed fragment masses. As such the structures of viequeamide E and F are unresolved at these residue positions.

The viequeamides were evaluated for their cytotoxic properties using the H460 human lung cancer cell line. Surprisingly, while the viequeamides are structurally quite similar to one another, they showed dramatically different biological activities. Viequeamide A (**1**) was observed to be potently cytotoxic with an $\text{LD}_{50} = 60 \pm 10$ nM, whereas a mixture of the minor compounds viequeamides B–F (**2–6**; however, dominated by compound **2** as noted prior) showed no toxicity relative to control at the maximum dose evaluated (30 $\mu\text{g/mL}$).

The viequeamides are structurally related to kulolide, a metabolite originally isolated by the Scheuer group from a predatory opisthobranch mollusk.¹⁰ Since that first report, there has been an increasing number of compounds reported of a related structural nature, we designate here these compounds as the “kulolide superfamily” which is recognizable by virtue of a β -hydroxy octanoic acid derivative along with a well defined sequence of amino acid or hydroxy acid residues in an overall cyclic arrangement. This superfamily can be subdivided into two groups: one containing the Dhoya residue, or its more reduced equivalents, Dhoca, and Dhooa, and the other possessing a Hmoya residue, or its brominated (Br-Hmoya) or reduced analogs, 3-hydroxy-2-methyl-7-octenoic acid (Hmoea), and 3-hydroxy-2-methyl-7-octanoic acid (Hmoaa). The structural trends between these subgroups are strongly conserved with the main exception being that the Hmoya subgroup always possesses a sequence of six residues, whereas the Dhoya subgroup usually contains one additional residue.

Within the kulolide superfamily the residue sequence can be summarized as: PKS residue (the presumed initiation of the biosynthetic pathway), valine, *N*-methylated non-polar amino acid, hydroxy acid analog of a non-polar amino acid, proline, non-polar amino acid which is often methylated at the amide nitrogen, and either another non-polar amino acid as in the case of the Dhoya subgroup or no further residues in the Hmoya subgroup. The most common non-polar amino acids observed are valine, isoleucine, and phenylalanine, and their modified *N*-methylated analogs, or the hydroxy acid analogs, 2-hydroxyisovaleric acid (Hiva), Hmpa, and phenyl lactic acid, respectively. A polar residue is only found in one example, the threonine of viequeamide A, and thus may explain its exceptional cytotoxicity relative to other members of the class. The greatest variability in residues is for those furthest from the PKS-derived portion, which is intriguing as these are predicted to be the final residues of the mixed PKS-NRPS biosynthetic assembly process, and thus may represent the most recently introduced modules from an evolutionary perspective (e.g. a possible neofunctionalization of a duplicated NRPS module). For example, the third residue in the Dhoya subgroup is variable with either an *N*-methyl valine or an *N*-methyl phenylalanine residue whereas the final and seventh residue is alternately glycine, alanine, valine or threonine (Figure 3, Table 3).

There are many cyclic depsipeptides containing a Dhoya, Hmoya, or Dhoya/Hmoya analog residues that differ from the sequence of residues that defines the kulolide superfamily. Examples include the Dhoya containing mantillamide²⁶ and the wewakpeptins,²⁷ which are larger than the members of the Dhoya subgroup, and the yanucamides^{28,29} and the cocosamides³⁰ which are shorter than the seven residue chain length common to the Dhoya subgroup. Geogamide³¹ is an interesting case in that it would match well to the residue sequence trends observed in the Dhoya subgroup, but only if the sequence of residues was reversed in its order.

Ultimately, while trends can be deduced from the sequence and overall architecture of these metabolites, and we thus are able to infer associations between them, a compelling knowledge of their evolutionary relationships will require access to their biosynthetic genes and comprising nucleotide sequence information. For example, the dudawalamides are also a notable case because dudawalamide A and B do not fit into the kulolide superfamily, while dudawalamide C–E do.¹⁵ Dudawalamide A has seven residues, as with most of the Dhoya Subgroup, but matches poorly to the trends in the residues observed for the kulolide superfamily. The sequence of the first three residues after the Dhoya residue is “lactic acid, alanine, *N*-methyl isoleucine”. This does not match the trend of “amino acid (almost exclusively valine), *N*-methyl amino acid, hydroxy acid” which is observed in the rest of the Dhoya subgroup. In dudawalamide B the structure of cyclo-[Dhoya-Val-*N*-Me-Phe-Pro-*N,O*-Me-Tyr-Ala] is one residue shorter than is common in other compounds in the Dhoya subgroup. If a hydroxy acid were added before the proline residue, it would fit well within the kulolide superfamily. Likewise, if the lactic acid residue were moved to before the proline in dudawalamide A, then this compound would also fit well within the kulolide superfamily. Thus, while a possible conjecture is that dudawalamide A and B are members of the kulolide superfamily wherein the biosynthetic pathway has been modified by a rearrangement and a deletion, respectively, of the hybrid PKS-NRPS biosynthetic pathway, without sequence data for these biosynthetic genes, this conjecture is provocative, yet unsubstantiated.

The sources of metabolites in the kulolide superfamily are diverse, both in terms of geographical location and the nature of the organisms from which the compounds were first isolated. However, the fact that the kulolide superfamily bears the structural hallmarks of filamentous prokaryotic secondary metabolites as well as the actuality of isolation of this compound superfamily directly from benthic cyanobacteria, strongly suggests that the

biosynthetic origin of all members of this superfamily derives from filamentous prokaryotes. The kulolides, kulomo'opunalides, and kulokainalide-1 were all obtained from the predatory mollusk *Philinopsis speciosa* from Oahu; however, because of the distinctive structural features of these compounds, the Scheuer group drew the inference at that time that *P. speciosa* was likely accumulating these compounds through its diet of sea hares that in turn fed on cyanobacteria.^{10,11} All other members of the kulolide superfamily were isolated directly from filamentous cyanobacteria, reported variably as *Lyngbya majuscula* (*Moorea producents*),^{12–19,32} *Moorea* sp.,¹⁵ *Symploca* cf. *hydroides*,²⁰ and *Oscillatoria margaritifera*.²¹ However, these identifications, except for the identification of *Oscillatoria margaritifera* as the producer of veraguamides A–C and H–L, were based on morphology and not 16S rRNA phylogenetic analysis. Considering the fact that the genera *Oscillatoria* and *Moorea* (previously *Lyngbya*) are morphologically very similar to one another, identifications based solely on this characteristic should be considered quite tenuous.^{21,32,33}

The cyanobacterial strain producing the viequeamides was analyzed for its phylogenetic relationship to other cyanobacteria using the 16S rRNA gene sequence. This analysis revealed that it possessed a closer relationship to members of the family Nostocaceae than Oscillatoriaceae. Unfortunately, as with genera in the family Oscillatoriaceae, overreliance on morphological characteristics has led some strains of *Calothrix*, *Rivularia*, *Tolypothrix*, and their closely related genera in the family Nostocaceae to be misidentified.^{34–36} In addition nutrient availability can produce morphological differences within a strain, further complicating identification of material collected from natural habitats when compared to culture collection strains grown under culture conditions.³⁷

The 16S rRNA phylogenetic analysis of this viequeamide-producing strain revealed that it aligned best with the genus *Rivularia*. This analysis further indicated that the two closely related genera *Calothrix* and *Rivularia* formed separate clades (see Supporting Information S29 and S30). The *Calothrix* clade was well supported with the exception of three sequences reported as *Rivularia*, EU009147, EU009149, and EU009150 (which showed 100% co-identity of residues for overlapping portions of their sequences). However, a prior report has suggested that the sequence EU009149 was incorrectly identified, and pertains to *Calothrix*, which is in good agreement with the analysis presented here.³⁵ The *Calothrix* strains that fell outside of the *Calothrix* clade were most likely misidentified as *Calothrix*, probably as a result of a morphology based identification.

The *Rivularia* clade contained strains alternately reported as *Calothrix*, unclassified Nostocales strains, as well as the uncultured 'button' cyanobacterium previously reported as *Dichothrix* sp. from thrombolites in Highborne Cay, Bahamas.³⁸

The viequeamide producer clustered robustly to the *Dichothrix* sp. strains, but together these strains did not separate from the *Rivularia* clade. *Dichothrix* is a poorly represented genus in culture collections and among published 16S rRNA sequences, a confounding factor in this phylogenetic analysis of the viequeamides producer. However, with the viequeamides producing strain and the *Dichothrix* strains robustly falling within the *Rivularia* clade, this strongly suggests that all of these organisms should be assigned to the genus *Rivularia* and not to *Dichothrix*.

Most members of the kulolide superfamily were isolated from collections made in the Pacific Ocean: Oahu in Hawaii,^{10,11} Thailand,¹⁷ Papua New Guinea,¹⁵ the Pacific coast of Panama,²¹ Singapore,^{18,19} and Guam.^{12,13,20} The veraguamides were isolated from both the Eastern and Western regions of the Pacific Ocean (Coiba, an island on the Pacific side of Panama and Guam).^{20,21} However, the antanapeptins and radamamides were isolated from Madagascar in the Indian Ocean.^{14,16} The isolation of the viequeamides from the Caribbean

Sea (Atlantic Ocean) represents the first members of the kulolide superfamily to be isolated from this ocean. Clearly, the kulolide superfamily of metabolites is distributed pan-tropically and thus likely represents an ancient biosynthetic pathway present in many different lineages of cyanobacteria.

A final point to note is the remarkable difference in biological properties within these newly isolated viequeamide natural products. Variation in biological properties has been previously noted in other members of the kulolide superfamily, such as the veraguamides.^{20,21} However, a comprehensive understanding of the structural features dictating these varying biological properties is not possible now because different protocols and cell lines have been used in the various studies of the biological properties of the kulolide family of compounds, and the relevant biological target or targets have not yet been defined. This latter area seems an especially promising area for investigation and efforts are underway to discover the cytotoxicity-related biological target of viequeamide A.

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotation was measured on a Jasco P-2000 Polarimeter. UV/visual-light spectra were measured on a Beckman Coulter DU 800 Spectrophotometer. IR spectra were measured on a Thermo Electron Corporation Nicolet IR 100 FT-IR. NMR spectra were collected on a Varian Unity 500 MHz (500 MHz and 125 MHz for the ¹H and ¹³C nuclei respectively) using CDCl₃ from Cambridge Isotope Laboratories, Inc. 99.8% D containing 0.03% v/v trimethylsilane (δ_{H} 0.0 and δ_{C} 77.16 as internal standards using trimethylsilane and CDCl₃, respectively). Microwave heated reactions were run in a Biotage Initiator microwave synthesizer. LCMS data for stereochemical analysis of the hydrolysates of **1** and **2** were obtained with a Phenomenex Luna 5 μm C18(2) 100Å column (4.6 $\times$ 250 mm) with a Thermo Finnigan Surveyor Autosampler-Plus/LC-Pump-Plus/PDA-Plus system and a Thermo Finnigan LCQ Advantage Max mass spectrometer. The elution for this analysis began with 10% CH₃CN/90% H₂O acidified with 0.1% v/v formic acid, and immediately ramped to 50% CH₃CN/50% H₂O acidified with 0.1% formic acid over 85 min at a flow rate of 0.4 mL/min (monitoring 200–600 nm and *m/z* 250–2000 in both positive and negative ion mode). LC-HRMS data for analysis of compounds **2–6** were obtained on an Agilent 6239 HR-ESI-TOFMS with a Phenomenex Luna 5 μm C18(2) 100Å column (4.6 $\times$ 250 mm) and a gradient starting at 60% CH₃CN/40% H₂O and immediately ramping to 100% CH₃CN over 20 min, then holding at 100% CH₃CN for 5 min. GCMS conducted with a Thermo Electron Corp. DSQ/TRACE-GC-Ultra GCMS system with a Cyclosil B column (Agilent Technologies J&W Scientific, 30 m $\times$ 0.25 mm). MS fragmentation experiments were run with a Biversa Nanomate (Advion Biosystems, Ithaca, NY) electrospray source for a Finnigan LTQ-FTICR-MS instrument (Thermo-Electron Corporation, San Jose, CA) running Tune Plus software version 1.0. HPLC purification was carried out with a Waters 515 HPLC Pump with a Waters 996 Photodiode Array Detector using Empower Pro software. All solvents were HPLC grade except for 99.8% acetone from Fisher which was distilled before use, and water which was purified by a Millipore Milli-Q system before use.

Collection and Identification of Cyanobacteria

Small 1–2 cm brown cyanobacterial puffballs were found growing in 0.5–1.5 m of water adhered to coral rubble, eelgrass, and dead gorgonian soft corals at Playa de la Chiva on Vieques Island in the Commonwealth of Puerto Rico, USA. A 1 L sample was preserved in 1:1 isopropanol/seawater solution, transported back to our laboratory in San Diego and stored at –20 °C until it was extracted. A small sample of cyanobacterial biomass was also persevered in RNA stabilization reagent (RNA^{later}™ from QIAGEN) for subsequent 16S

rRNA sequencing and analysis. A voucher sample was stored in our laboratory (VQC-26/MAR/11-1).

Polymerase Chain Reaction (PCR) and Cloning

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Inc., Madison, WI, USA) following the manufacturer's specifications. DNA concentration and purity were measured on a DU® 800 spectrophotometer (Beckman Coulter). The 16S rRNA genes were PCR-amplified from isolated DNA using the cyanobacterial specific primers 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 809R 5'-GCTTCGGCACGGCTCGGGTCGATA-3'. The PCR reaction contained 1.0 μ L (~100 ng) of DNA, 2.5 μ L of 10 $\times$ PfuUltra IV reaction buffer, 1.0 μ L (10 mM) of dNTP mix, 1.0 μ L of each primer (10 μ M), 1.0 μ L of PfuUltra IV fusion HS DNA polymerase and 17.5 μ L of water for a total volume of 25 μ L. The PCR reactions were performed in an Eppendorf® Mastercycler® gradient as follows: initial denaturation for 4 min at 95 °C, amplification by 30 cycles of 30 sec at 95 °C, 30 sec at 50 °C and 1 min at 72 °C, and final elongation for 7 min at 72 °C. PCR products were purified using a MinElute® PCR Purification Kit (Qiagen) before subcloning with the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) following the manufacturer's specifications. Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen) and sequenced with M13 primers. The 16S rRNA gene sequence is available in the DDBJ/EMBL/GenBank databases under acc. No. JQ979179.

Phylogenetic Inferences

All gene sequences were analyzed using Geneious Pro v.5.5.4.³⁹ The 16S rRNA gene sequences were aligned using the L-INS-I algorithm in MAFFT v6.814b.⁴⁰ Best-fitting nucleotide substitution models optimized by maximum likelihood (ML) selected using corrected Akaike/Bayesian Information Criterion (AIC/BIC) in jModelTest v0.1.1.⁴¹ The evolutionary histories of the cyanobacterial genes were inferred using ML and Bayesian inference algorithms. The ML inference was performed using PhyML⁴² in Geneious Pro v5.5.4. The analysis was run using the GTR+I+G model (selected by AIC and BIC criteria) assuming heterogeneous substitution rates and gamma substitution of variable sites (proportion of invariable sites (pINV) = 0.067, shape parameter (α) = 0.275, number of rate categories = 4). Bootstrap resampling was performed on 1,000 replicates. Bayesian analysis was conducted using MrBayes⁴³ in Geneious Pro v5.5.4 with four Metropolis-coupled MCMC chains (one cold and three heated) ran for 3,000,000 generations. The first 10% were discarded as burn-in and data set was sampled with a frequency of every 200 generations. The molecular clock parameters were set for uniform branch lengths, tree height exponential = 1 and shape parameter exponential = 10.

Cytotoxicity Assay

H460 cells were added to 96-well plates at 3.33×10^4 cells/mL of Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells, in a volume of 180 μ L per well, were incubated overnight (37 °C, 5% CO₂) to allow recovery before treatment with the test compounds. Compounds were dissolved in dimethylsulfoxide to a stock concentration of 10 mg/mL. Working solutions of the compounds were made in RPMI 1640 medium without FBS, with a volume of 20 μ L added to each well to give a final compound concentration of either 30 or 3 μ g/mL. An equal volume of RPMI 1640 medium without FBS was added to wells designated as negative controls for each plate. Plates were incubated for approximately 48 h before staining with Thiazolyl Blue Tetrazolium Bromide (98% Sigma). Using a ThermoElectron Multiskan Ascent plate reader, plates were read at 570 and 630 nm. Concentration response graphs were generated using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Extraction and Isolation

The preserved biomass/alcohol solution was filtered through cheese cloth and the crude algal biomass extracted by soaking for 15 min in 600 mL of 2:1 CH₂Cl₂/MeOH with manual fragmentation of the cyanobacterial clumps. The organic layer was then filtered through cheese cloth and the algal biomass was repetitively re-extracted six times by submerging in 2:1 CH₂Cl₂/MeOH with mild warming (< 30 °C) for 30 min and then filtering through cheese cloth. The aqueous layer was concentrated to a reduced volume (ca. 500 mL) by rotary evaporation (< 35 °C) and then extracted with an equal volume of CH₂Cl₂ using 30 mL of brine to assist in phase separation. All of the organic layers were then combined and concentrated by rotary evaporation to afford 6.6 g of black-green solids. A small portion was preserved for future bioassays and the remainder was purified by VLC with 300 mL of silica gel (Type H, 10–40 µm, Sigma-Aldrich) in a 10 cm diameter by 9 cm glass vacuum armed frit; fractions of 300 mL volume using progressively more polar mixtures of hexanes–EtOAc–MeOH (nine fractions, 100% hexanes, 10% EtOAc/90% hexanes, 20% EtOAc/80% hexanes, then 20% increments to 100% EtOAc, 25% MeOH/75% EtOAc, and 100% MeOH).

The 100% EtOAc eluting fraction was concentrated by rotary evaporation to afford 0.245 g of a dark brown-orange oil. This material was purified by RP-SPE with a Strata 6 mL column with 1 g of C18-E (55 µm, 70 Å) packing material prepared with five column washes of CH₃CN followed by five column volumes of 50% CH₃CN/50% H₂O. The material from the EtOAc fraction was dissolved in 50% CH₃CN/50% H₂O, loaded onto the column, and six fractions obtained using 30 mL each of 50% CH₃CN/50% H₂O, 60% CH₃CN/40% H₂O, 80% CH₃CN/20% H₂O, CH₃CN, MeOH, and CH₂Cl₂. The 50% CH₃CN/50% H₂O fraction was sequentially placed in 3 mL of EtOAc and 3 mL of 75% CH₃CN/25% H₂O, the insoluble material removed by filtration, and the filtrate purified by RP-HPLC using a Synergi Fusion RP-80 (250 × 10.00 mm, 4 µm, isocratic elution with 60% CH₃CN/40% H₂O at 3 mL/min over 70 min). This afforded 14.8 mg of a white solid containing ca. 92% pure viequeamide A (**1**) (purity by ¹H NMR integration) which was used for NMR-based structure elucidation. Re-purification of this material by RP-HPLC using the same conditions gave 13.6 mg of a white solid which had no detectible impurities by NMR, but trace **2** when using selected ion chromatogram LCMS; this sample was used for bioassays and measurement of optical rotation.

The insoluble matter from the EtOAc and 75% CH₃CN/25% H₂O solubilization steps contained compounds similar to viequeamide A by ¹H NMR and LCMS. These materials were combined, dissolved in pure CH₃CN, and purified by RP-HPLC using a Synergi Fusion RP-80 (250 × 10.00 mm, 4 µm column, isocratic elution of 2:1 CH₃CN/H₂O elution at 3 mL/min over 40 min). The HPLC fractions from this purification were still impure; however, LCMS and ¹H NMR analyses suggested the presence of a mixture of interconverting *N*-methyl amide rotomers that caused broad and overlapping elution peaks, thus yielding impure mixtures. Several fractions from this latter HPLC were combined to yield a simplified mixture with a single major compound (> 95% compound **2**, by ¹H NMR), and this was analyzed by 2D NMR and mass spectrometry to provide the planar structure of viequeamide B. The remaining uncombined fractions from the latter HPLC work (0.5 mg) contained a mixture of compounds **2–6** in which **3** and **4** predominated.

Viequeamide A (**1**)

white amorphous solid; [α]_D²³ –32.6 (CH₂Cl₂); UV (MeOH) λ_{max} (log ε) 204 (4.30) nm; IR (neat) ν_{max} 3387, 2967, 1749, 1641, 1455, 1366, 1308, 1154, 1122, 733 cm^{–1}; see Table 1 for NMR data; HR-ESI-TOF-MS [M + Na]⁺ *m/z* 826.4940 (calculated for C₄₂H₆₉N₅O₁₀Na 826.4937).

Viequeamide B (2)

white amorphous solid; see Table 2 for NMR data; HR-ESI-TOF-MS $[M + Na]^+$ m/z 830.4670 (calculated for $C_{44}H_{65}N_5NaO_9$ 830.4674).

LC-HRMS Fragmentation Analysis of Viequeamides B–F (2–6)

Liquid chromatography-mass spectrometry of a purified fraction containing viequeamides B–F (2–6) revealed four major masses at m/z 830.4666, m/z 832.4833, m/z 834.4988, and m/z 844.4833, corresponding to molecular formulas $C_{44}H_{65}N_5NaO_9$, $C_{44}H_{67}N_5NaO_9$, $C_{44}H_{69}N_5NaO_9$, and $C_{45}H_{67}N_5NaO_9$, respectively. To characterize the planar structures of 3–6, a separate experiment was conducted to fragment the major masses observed in the LC-HRMS analysis. The data were obtained on these four m/z values from two separate HPLC fractions at ca. 0.1 mM (CH_3CN), one of which was from HPLC fractions dominated by compound 2, and the other from HPLC fractions dominated by 3 and 4. A 50% MeOH/50% H_2O solution was acidified with 1% v/v formic acid (Acros Organics 98+ %) and used to dilute 10-fold the 0.1 mM samples (e.g. 1 μL of sample into 10 μL acidified MeOH/ H_2O).

Samples were subjected to electrospray ionization on the Nanomate nano-spray source (pressure: 0.3 psi, spray voltage: 1.4kV), then fragmented and analyzed on the LTQ-FT-MS. Generally, the instrument was first auto-tuned on the m/z value of the ion to be fragmented. Then, the $[M + H]^+$ ion of each compound was isolated in the linear ion trap and fragmented by collision induced dissociation. Sets of consecutive, high-resolution MS/MS scans were acquired in profile mode and averaged using QualBrowser software by Thermo (see Supporting Information S16–19).

Stereochemical Analysis of Viequeamide A (1) by Marfey's Analysis and Chiral GCMS

A 60 μL aliquot (600 μg) was transferred from a 10 mg/mL solution of viequeamide A (1) in CH_3CN to a conical microwave reaction tube with a stir bar. The solvent was removed under N_2 (g) flow and the resulting solid was dissolved in 600 μL of 6 M HCl (aq). This solution was stirred and heated by microwave to 160 $^{\circ}C$ for 5 min, and the resulting material was divided into two equal portions and both were dried under N_2 (g) flow.

One portion was dissolved in 0.75 mL of a 1 M $NaHCO_3$ (aq) solution, and to this was added drop wise 71 μL of 1-fluoro-2,4-dinitrophenyl-D-alanine amide (D-FDAA) in acetone (1.0 mg/mL), incubated for 1 h at 35–45 $^{\circ}C$, and then quenched with 370 μL of 2 M HCl (aq). The reaction mixture was transferred to fresh vial using CH_3CN/H_2O mixtures, reduced in volume under N_2 (g) flow, and diluted to ca. 1 mL with 50% CH_3CN /50% H_2O ; 10 μL of this mixture was analyzed by HPLC-ESIMS comparison to authentic amino acid standards, all D-FDAA derivatized. Retention times for the authentic standards were as follows: D-Val (62.48 min), L-Val (71.58 min), D-Thr (41.30 min), D-*allo*-Thr (41.46 min), L-Thr (48.60 min), L-*allo*-Thr (44.74 min), D/L-*N*-Me-Val (69.92 and 74.73 min), L-*N*-Me-Val (74.70 min), D-Pro (51.55 min), and L-Pro (54.53 min). The hydrolysate peaks with the expected masses were found at 71.82, 48.79, 74.91, and 54.53 min which correspond to L-Val, L-Thr, L-*N*-Me-Val, and L-Pro.

The second portion of the acid hydrolysis product was dissolved in 0.6 mL of 1:1 Et_2O /MeOH, and CH_2N_2 in Et_2O was added by polished glass pipette until the solution was a persistent yellow color. The reaction mixture was allowed to stand for 0.5 h at room temperature and then dried under N_2 (g) flow. The resulting material was dissolved in ca. 12 μL of CH_2Cl_2 and 1 μL was injected into the GCMS system at 35 $^{\circ}C$ for 15 min followed by a ramp of 1.5 $^{\circ}C$ /min until 60 $^{\circ}C$ was reached at which time the GC was ramped at 50 $^{\circ}C$ /min until 170 $^{\circ}C$ where the temperature was held for 5 min. (For all GCMS runs, the ion source was held at 250 $^{\circ}C$ and the positive ion scan from 50 to 1000 m/z was collected from

7 min until the end of the run.) Retention times of previously prepared authentic standards,²¹ were measured for 2*S*,3*S*-Hmpa, 2*S*,3*R*-Hmpa, 2*R*,3*R*-Hmpa, and 2*R*,3*S*-Hmpa (40.4, 39.4, 40.0, and 39.8 min respectively) and compared with that of the natural product hydrolysate (39.8 min) showing the residue in viequeamide A to be 2*R*,3*S*-Hmpa. Coinjections of the standards and the natural product hydrolysate confirmed this result.

To determine the configuration of the stereocenter in the Dhoya moiety, a stirred solution containing 400 µg of **1** in 400 µL of EtOH was treated with 1.25 mg of 10% palladium on carbon, and the atmosphere was replaced with H₂ (g) by balloon. The reaction mixture was stirred at room temperature for 3 h and filtered through glass wool into a microwave reaction tube with a stir bar, with ca. 2 mL CH₂Cl₂. The sample was then concentrated under N₂ (g) flow, redissolved in 800 µL of 2 M HCl (aq), and the resulting mixture stirred and heated by microwave at 160 °C for 5 min. The reaction mixture was partially dried under N₂ (g) flow and the remaining residue of water removed via azeotrope with 2 mL of benzene. The resulting solids were dissolved in 400 µL of 1:1 Et₂O/MeOH, and while stirring CH₂N₂ in Et₂O was added in excess. After 1.25 h significant color loss was observed, and thus another portion of CH₂N₂ in Et₂O was added and stirring was continued for 45 min. The reaction mixture was concentrated under N₂ (g) flow and the residue quickly dissolved in CH₂Cl₂, transferred to a GCMS vial, and re-concentrated under N₂ (g) flow to ca. 12 µL total volume.

Chiral GCMS analysis (Cyclosil B) of the above product and synthetic Dhoya standards based on a method recently described.²⁴ Briefly, the initial temperature was 40 °C with an immediate ramp of 20 °C/min to 100 °C where upon the temperature was held for 4 min. The oven temperature was then again ramped at 2 °C/min until 130 °C was reached where upon the temperature was held for 5 additional min. The retention times of both the *S* and the *R* standards (24.3 and 24.5 min, respectively) were measured and compared with that of the natural product hydrolysate (24.3 min), thus revealing the presence of an *S*-Dhoya unit in viequeamide A. Coinjections of standards and the natural product hydrolysate confirmed this result.

Stereochemical Analysis of Viequeamide B (**2**) by Marfey's Analysis and Chiral GCMS

A 700 µg sample of **2** (from HPLC described above, minor analogs **3–6** present but mixture dominated by **2** by ¹H-NMR) was dissolved in 700 µL of ethanol, treated with 2.0 mg of 10% palladium on carbon, and the atmosphere replaced with H₂ (g) by balloon. The reaction mixture was stirred at room temperature for 1 h and filtered through glass wool with 3 × 0.5 mL of CH₂Cl₂. The filtrate was concentrated to dryness by rotory evaporation, and transferred to a microwave reaction tube with a stir bar, again through glass wool with 3 × 0.5 mL of CH₂Cl₂. The sample was concentrated under N₂ (g) flow, redissolved in 700 µL of 2 M HCl (aq), and the resulting mixture was stirred and heated by microwave at 160 °C for 5 min. The reaction mixture was then concentrated to dryness under N₂ (g) flow, and the resulting solids were dissolved in 700 µL of CH₂Cl₂ and partitioned into a 400 µL and 300 µL sample.

The 300 µL sample was concentrated to dryness in a fresh vial and redissolved in 300 µL of 1 M NaHCO₃ (aq), and a stir bar and 1.0 mL of 1-fluoro-2,4-dinitrophenyl-5-L-valine amide (L-FDVA) in acetone (at a concentration of 1.0 mg/mL) was added. The valine amide analog was selected because in testing the L-FDAA derivatized authentic *N*-Me-Ala standards, no separation was observed between diastereomers despite lengthening the elution gradient of the HPLC method. The reaction mixture was then stirred at 30 °C for 1 h, and then quenched with 150 µL of 2 M HCl (aq). The reaction mixture was then transferred to a fresh vial with 3 × 1.0 mL of CH₃CN through an Advantec syringe filter (HP020AN), concentrated to dryness under N₂ (g) flow, and transferred to an LCMS vial again through a

syringe filter with 3×0.5 mL of CH_3CN ; 10 μL of this mixture was analyzed by HPLC-ESIMS comparison to authentic amino acid standards, all L-FDVA derivatized. Retention times for the authentic standards were as follows: D/L-*N*-Me-Ala (76.85 and 77.91 min), L-*N*-Me-Ala (76.80), D-Val (101.73 min), D/L-Val (82.39 and 101.88 min), D/L-*N*-Me-Val (91.64 and 103.87 min), L-*N*-Me-Val (91.69 min), D-Pro (79.10 min), and L-Pro (70.58 min). The hydrolysate peaks with the expected masses were found at 76.40, 82.24, 91.69, and 70.59 min which correspond to L-*N*-Me-Ala, L-Val, L-*N*-Me-Val, and L-Pro.

The 400 μL sample was concentrated to dryness redissolved in 400 μL of 1:1 $\text{Et}_2\text{O}/\text{MeOH}$ and while stirring, CH_2N_2 in Et_2O was added in excess. After 1 h significant color loss was observed, and thus another portion of CH_2N_2 in Et_2O was added, and stirring continued for 1 h. The reaction was then concentrated to dryness under N_2 (*g*) flow and the residue quickly transferred to a GCMS vial with 5×50 μL of CH_2Cl_2 .

Chiral GCMS analysis (Cyclosil B) of the above product and synthetic Dhoya standards was performed with the identical method used for the hydrolysate of **1**. The retention times of the *S* and *R* standards (23.7 and 23.9 min, respectively) were measured and compared with that of the natural product hydrolysate (23.7 min), thus revealing the presence of an *S*-Dhoya unit in viequeamide B. Coinjections of standards and the hydrogenated natural product hydrolysate confirmed this result.

A separate method was used to compare the hydrolysate to commercially available standards for Pla. Using the same column as above the initial temperature of 40 °C was held steady for 5 min, then ramped at 5 °C/min to 100 °C, then immediately ramped to 130 °C at 1.5 °C/min. The retention times of the *S* and *R* standards (44.78 and 43.45 min, respectively) were measured and compared with that of the natural product hydrolysate (45.00 min), thus revealing the presence of an *S*-Pla unit in viequeamide B. Coinjections of standards and the hydrogenated natural product hydrolysate confirmed this result.

While compounds **3–6** were present in this mixture, because of their low concentration in the sample it is unlikely that products from the hydrolysis of **3–6** could be detected if they differed from the major peaks from **2**, and thus the stereoconfiguration of **3–6** must still be considered unresolved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Key 2D-NMR correlations of viequeamide A (1) and viequeamide B (2).

Figure 2.
MS₂ fragment ions for the isobaric compounds viequeamide E (5) and F (6).

Figure 3.

The prevalence of residues found in the kulolide superfamily. (Size of the font is proportional to the prevalence of those residues within the family. The residues in boxes are shown at twice their proportional size to enhance readability.) ^aLac, lactic acid. ^bIn kulokainalide-1 there is a unique eighth residue, phenylalanine.¹¹ ^c While shown as isoleucine, these residues were determined by fragmentation analysis and are unresolved between leucine, isoleucine, or *N*-methyl valine as noted in the text (see Figure 2, and S19).

Table 1

Summary of NMR data (in CDCl₃) for viequeamide A (**1**).

Residue	Position	δ_C , type	δ_H (J in Hz)	HMBC	TOCSY	ROESY
Thr	1	169.2, C	–			
	2	58.1, CH	4.68, dd (10.3, 3.2)	1, 3, 5	Thr-NH, 3	
	3	68.2, CH	3.87, m		Thr-OH, 2, 4	
	4	19 ^a , CH ₃	0.80, d (6.4)	2, 3		
N-Me-Val-1	OH	–	4.48, d (12.4)	3	3, 4	Thr-NH
	NH	–	8.04, d (10.4)	5	2	Thr-OH, 6
	5	168.2, C	–			
	6	67.4, CH	4.06, d (12.4)	5, 7, 10, 11	7, 8, 9	Thr-NH, 12
	7	26.0, CH	2.41, m	6	6, 8, 9	
	8	19 ^a , CH ₃	0.84, d (6.7)	6, 7, 9	6, 7, 9	
	9	20.1, CH ₃	0.93–1.00 ^b			
Pro	10	29.0, CH ₃	2.77, s	6, 11		7
	11	172.1, C	–			
	12	55.7, CH	5.01, dd (8.4, 3.1)		13a/b, 14, 15a/b	6
	13a	29.7, CH ₂	2.09, m		12, 13b, 14, 15a/b	
	13b		2.03, m		12, 13a, 14, 15a/b	
	14	25.4, CH ₂	2.52, m		12, 13a/b, 15b,	
	15a	47.4, CH ₂	3.95, m	13, 14	12, 13a/b 15b	15b, 17
	15b		3.52, m	13, 14	12, 13a/b, 14, 15a,	15a, 17
Hmpa ^c	16	168.7, C	–			
	17	74.5, CH	4.82, d (2.0)	18–21 weak		15a/b
	18	36.6, CH	1.72, m	19, 20, 21		
	19	13.8, CH ₃	1.10, d (6.7)	18, 20, 21		
	20	27.3, CH ₂	1.43–1.55 ^d	18, 20, 21		
	21	12.0, CH ₃	0.93–1.00 ^b			
N-Me-Val-2	22	170.3, C	–			
	23	63.7, CH	3.91, d (10.7)	22, 24, 27, 28	24, 25, 26	29

Residue	Position	δ_C , type	δ_H (J in Hz)	HMBC	TOCSY	ROESY
Val	24	29.5, CH	2.28 ^e		23, 25, 26	
	25	19 ^a , CH ₃	0.93–1.00 ^b			
	26	19 ^a , CH ₃	0.93–1.00 ^b			
	27	28.3, CH ₃	2.98, s	23, 28		
	28	172.8, C	–			
	29	53.9, CH	4.89, dd (7.3, 2.3)	28, 30, 33	Val-NH	23
	30	31.7, CH	1.96, m		31, 32	
	31	20.7, CH ₃	0.93–1.00 ^b			
	32	16.1, CH ₃	0.75, d (6.6)	29, 30, 31	30, 31	
	NH	–	6.83, d (7.4)	33	29	35
Dhoya	33	174.7, C	–			
	34	46.7, C	–			
	35	17.1, CH ₃	1.36, s	33, 34, 36, 37		Val-NH
	36	25.6, CH ₃	1.18, s	33, 34, 35, 37		
	37	77.4, CH	5.55, d (8.5)	1, 33, 34	38, 39, 40	
	38a	27.9, CH ₂	1.76, m			
	38b		1.43–1.55 ^d			
	39	24.8, CH ₂	1.43–1.55 ^d	41		
	40	18.1, CH ₂	2.19–2.32 ^e	41, 42	37, 38, 39, 42	
	41	84.2, C	–			
	42	68.9, CH	1.93, t (2.6)		38, 39, 40	

^aThe carbon resonances at 18.9, 19.1, 19.4, and 19.6 were very close together and their HSQC associations could not be confidently assigned; HMBC correlations failed to confidently resolve their identities as well.

^bThese proton resonances overlapped.

^cHmpa, 2-hydroxy-3-methyl-pentanoic acid.

^{d,e}These proton resonances overlapped.

Table 2

Summary of NMR data (in CDCl₃) for viequeamide B (2).

Residue	Position	δ_C , type	δ_H (J in Hz)	HMBC	TOCSY	ROESY
Val-1	1	169.8, C	–			
	2	57.1, CH	4.77, dd (9.5, 3.9)	1, 3–6	Val-NH (A), 3–5	
	3	29.6, CH	2.31, m		Val-NH (A), 2, 4, 5	
	4	20.4, CH ₃	0.99, d (6.8)	2, 3, 5	Val-NH (A), 2, 3, 5	
	5	18.2, CH ₃	0.73, d (6.9)	2, 3, 4	Val-NH (A), 2–4	
NH (A)	–	–	7.57, d (9.6)	2, 6	2–5	9
N-Me-Ala	6	170.4, C	–			
	7	55.9, CH	4.28, q (7.0)	6, 8, 9	8	9, 11
	8	15.1, CH ₃	1.48, d (7.0)	7, 6	7	
	9	30 ^d , CH ₃	2.80, s ^b	7, 10		Val-NH (A), 7, 8
Pro	10	171.0, C	–			
	11	57.4, CH	4.17, m	12, 13	12, 13, 14	12, 13
	12	30.4, CH ₂	1.45–1.62, m ^c			
	13a	21.8, CH ₂	1.71, m ^d			
Pla	13b		1.54, m ^c			
	14a	45.9, CH ₂	3.61, m	12, 13	13, 14b	
	14b		3.24, m	12, 13, 15	13, 14a	
	15	167.0, C	–			
	16	70.9, CH	5.72, dd (11.3, 5.1)	15, 17, 18, 22	17a, 17b	11, 19
	17a	37.9, CH ₂	3.73, dd (11.8, 5.1)	15, 16, 18, 19	16	19
	17b		3.28, t (11.6)	15, 16, 18, 19	16	19
	18	135.7, C	–			
N-Me-Val	19	129.9, CH	7.42, d (7.4)	17, 20, 21	20, 21	11, 16, 17
	20	128.3, CH	7.28, m	18, 19	19, 21	
	21	126.9, CH	7.20, m	18, 19	19, 20	
	22	170.5, C	–			
	23	64.9, CH	4.48, d (9.0)	22, 24–26, 28	24–26	27, 29

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Residue	Position	δ_c , type	δ_H (J in Hz)	HMBC	TOCSY	ROESY
Val-2	24	30 ^a , CH	2.36–2.27, m	23	23, 25, 26	27
	25	19.9, CH ₃	1.18, d (6.7)	23, 24, 26	23, 24, 26	27
	26	21.3, CH ₃	1.03, d (6.7)	23–25	23–25	27
	27	30 ^a , CH ₃	2.99, s	23, 28		23–26
	28	172.6, C	–			
	29	53.5, CH	4.71, t (8.7)	28, 30–33	Val-NH (B), 30–32	23
	30	32.6, CH	2.02, m	29	29, 31, 32	
NH (B)	31	18.3, CH ₃	0.93, d (6.7)	29, 30, 32	Val-NH (B), 29, 30, 32	
	32	19.8, CH ₃	0.93, d (6.7)	29–31	Val-NH (B), 29–31	
		–	6.16, d (8.8)	28, 30–33	29–32	35
	33	174.7, C	–			
	34	46.2, C	–			
	35	17.0, CH ₃	1.26, s	33, 34, 36, 37		Val-NH (B)
	36	25.7, CH ₃	1.15, s	33–35, 37		37
Dhoya	37	77.9, CH	5.75, dd (11.3, 1.8)	1, 33–35, 39	38–40	
	38	27.5, CH ₂	1.71, m ^d		37, 39, 40, 42	
	39	24.6, CH ₂	1.45–1.63, m ^c		37, 38, 40, 42	
	40	17.9, CH ₂	2.23, dt (6.6, 2.5)	38, 39, 41, 42	37–39, 42	
	41	83.7, C	–			
	42	69.1, CH	1.95, t (2.5)		38–40	

^aTightly clustered carbon signals at 30.4, 30.3, 30.3 and 30.2 could not be resolved by HSQC or HMBC, although a DEPT-135 did reveal 30.4 to be the methylene carbon C-12 in proline.

^bMinor rotomer at 2.79, s.

^{c,d}Overlapping signals in the proton spectrum.

Table 3

Metabolites of the kulolide superfamily and their residue sequences.

Compound Name Residues		1 st	2 nd	3 rd	4 th	5 th	6 th	7 th
Dhoya Subgroup								
Kulolide-1 ^{10,11}	<i>S</i> -Dhoya	L-Val	D- <i>N</i> -Me-Val	<i>S</i> -Pla	L-Pro	L-Val	L-Ala	
Kulolide-2 ¹¹	<i>S</i> -Dhoea	L-Val	D- <i>N</i> -Me-Val	<i>S</i> -Pla	L-Pro	L-Val	L-Ala	
Kulolide-3 ¹¹	<i>S</i> -Dhoaa	L-Val	D- <i>N</i> -Me-Val	<i>S</i> -Pla	L-Pro	L-Val	L-Ala	
Kulokainalide-1 ¹¹	<i>S</i> -Dhoya	L-Val	D- <i>N</i> -Me-Val	<i>S</i> -Lac	L-Pro	L-Pro	L-Val-L-Phe ^a	
Ptiptetrolide A ¹²	<i>S</i> -Dhoya	L-Val	L- <i>N</i> -Me-Phe	2 <i>S</i> ,3 <i>S</i> -Hmpa	L-Pro	L-Ile	Gly	
Ptiptetrolide B ¹²	<i>S</i> -Dhoea	L-Val	L- <i>N</i> -Me-Phe	2 <i>S</i> ,3 <i>S</i> -Hmpa	L-Pro	L-Ile	Gly	
Ptiptetrolide C ¹³	<i>S</i> -Dhoaa	L-Val	L- <i>N</i> -Me-Phe	2 <i>S</i> ,3 <i>S</i> -Hmpa	L-Pro	L-Ile	Gly	
Ptiptetrolide D ¹³	<i>S</i> -Dhoya	L-Val	L-Phe	2 <i>S</i> ,3 <i>S</i> -Hmpa	L-Pro	L-Ile	Gly	
Ptiptetrolide E ¹³	<i>S</i> -Dhoya	L-Val	L- <i>N</i> -Me-Phe	<i>S</i> -Hiva	L-Pro	L-Ile	Gly	
Ptiptetrolide F ¹³	<i>S</i> -Dhoya	L-Val	L- <i>N</i> -Me-Phe	2 <i>S</i> ,3 <i>S</i> -Hmpa	L-Pro	L-Val	Gly	
Radamamide A ¹⁴	<i>S</i> -Dhoya	L-Val	L- <i>N</i> -Me-Val	2 <i>S</i> ,3 <i>S</i> -Hmpa	L-Pro	L- <i>N</i> -Me-Phe	Gly	
Dudawalamide C ¹⁵	Dhoya	Val	<i>N</i> -Me-Val	Hiva	Pro	<i>N</i> -Me-Phe	Gly	
Dudawalamide D ¹⁵	Dhoya	Val	<i>N</i> -Me-Val	Hmpa	Pro	<i>N</i> -Me-Phe	Gly	
Dudawalamide E ¹⁵	<i>S</i> -Dhoya	L-Val	L- <i>N</i> -Me-Phe	<i>S</i> -Hiva	L-Pro	L-Ile	Gly	
Viequeamide A	<i>S</i> -Dhoya	L-Val	L- <i>N</i> -Me-Val	2 <i>R</i> ,3 <i>S</i> -Hmpa	L-Pro	L- <i>N</i> -Me-Val	L-Thr	
Viequeamide B	<i>S</i> -Dhoya	L-Val	L- <i>N</i> -Me-Val	<i>S</i> -Pla	L-Pro	L- <i>N</i> -Me-Ala	L-Val	
Viequeamide C	Dhoea	Val	<i>N</i> -Me-Val	Pla	Pro	<i>N</i> -Me-Ala	Val	
Viequeamide D	Dhoaa	Val	<i>N</i> -Me-Val	Pla	Pro	<i>N</i> -Me-Ala	Val	
Viequeamide E	Dhoya	Ile ^b	<i>N</i> -Me-Val	Pla	Pro	<i>N</i> -Me-Ala	Val	
Viequeamide F	Dhoya	Val	<i>N</i> -Me-Val	Pla	Pro	<i>N</i> -Me-Ala	Ile ^b	
Hmoya Subgroup								
Kulomo'opunalide-1 ¹¹	2 <i>S</i> , 3 <i>R</i> -Hmoya	L-Val	L- <i>N</i> -Me-Ile	2 <i>S</i> ,3 <i>S</i> -Hmpa	L-Pro	L- <i>N</i> -Me-Ile	--	
Kulomo'opunalide-2 ¹¹	2 <i>S</i> , 3 <i>R</i> -Hmoya	L-Val	L- <i>N</i> -Me-Ile	<i>S</i> -Hiva	L-Pro	L- <i>N</i> -Me-Ile	--	
Antanapeptin A ¹⁶	Hmoya	L-Val	L- <i>N</i> -Me-Phe	<i>S</i> -Hiva	L-Pro	L- <i>N</i> -Me-Ile	--	
Antanapeptin B ¹⁶	Hmoea	L-Val	L- <i>N</i> -Me-Phe	<i>S</i> -Hiva	L-Pro	L- <i>N</i> -Me-Ile	--	

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Compound Name	Residues	1 st	2 nd	3 rd	4 th	5 th	6 th	7 th
Antanapeptin C ¹⁶	Hmoaa	L-Val	L-N-Me-Phe	S-Hiva	L-Pro	L-N-Me-Ile	--	--
Antanapeptin D ¹⁶	Hmoya	L-Val	L-N-Me-Phe	S-Hiva	L-Pro	L-N-Me-Val	--	--
Radamamide B ¹⁴	Hmoya	L-Ile	L-N-Me-Val	2S, 3S-Hmpa	L-Pro	L-N-Me-Ile	--	--
Trungapeptin A ¹⁷	2S, 3R-Hmoya	L-Val	L-N-Me-Val	S-Pla	L-Pro	L- <i>allo</i> -Ile	--	--
Trungapeptin B ¹⁷	2S, 3R-Hmoaa	L-Val	L-N-Me-Val	S-Pla	L-Pro	L- <i>allo</i> -Ile	--	--
Trungapeptin C ¹⁷	2S, 3R-Hmoaa	L-Val	L-N-Me-Val	S-Pla	L-Pro	L- <i>allo</i> -Ile	--	--
Hantupeptin A ^{18,19}	2R, 3S-Hmoya	L-Val	L-N-Me-Ile	S-Pla	L-Pro	L-N-Me-Val	--	--
Hantupeptin B ¹⁹	2R, 3S-Hmoaa	L-Val	L-N-Me-Ile	S-Pla	L-Pro	L-N-Me-Val	--	--
Hantupeptin C ¹⁹	2R, 3S-Hmoaa	L-Val	L-N-Me-Ile	S-Pla	L-Pro	L-N-Me-Val	--	--
Veraguamide A ^{20,21}	8-Br-2S, 3R-Hmoya	L-Val	L-N-Me-Val	2S, 3S-Hmpa	L-Pro	L-N-Me-Val	--	--
Veraguamide B ^{20,21}	8-Br-2S, 3R-Hmoya	L-Val	L-N-Me-Val	S-Hiva	L-Pro	L-N-Me-Val	--	--
Veraguamide C ^{20,21}	2S, 3R-Hmoya	L-Val	L-N-Me-Val	2S, 3S-Hmpa	L-Pro	L-N-Me-Val	--	--
Veraguamide D ²⁰	2S, 3R-Hmoya	L-Val	L-N-Me-Val	2S, 3S-Hmpa	L-Pro	L-N-Me-Ile	--	--
Veraguamide E ²⁰	2S, 3R-Hmoya	L-Ile	L-N-Me-Ile	2S, 3S-Hmpa	L-Pro	L-N-Me-Val	--	--
Veraguamide F ²⁰	2S, 3R-Hmoya	L-Val	L-N-Me-Val	S-Pla	L-Pro	L-N-Me-Val	--	--
Veraguamide G ²⁰	2S, 3R-Hmoya	L-Val	L-N-Me-Val	2S, 3S-Hmpa	L-Pro	L-N-Me-Val	--	--
Veraguamide H ²¹	Hmoya	Val	N-Me Val	Hiva	Pro	N-Me Val	--	--
Veraguamide I ²¹	Hmoaa	Val	N-Me Val	Hmpa	Pro	N-Me Val	--	--
Veraguamide J ²¹	Hmoaa	Val	N-Me Val	Hiva	Pro	N-Me Val	--	--
Naopeptin ¹⁵	Hmoya	Val	N-Me-Val	Pla	Pro	N-Me-Ile	--	--

^a Kulokainalide-1 is eight residues long, rather than the seven seen in most of the Dhoya Subgroup.

^b While shown as isoleucine, these residues were determined by fragmentation analysis and are unresolved between leucine, isoleucine, or N-methyl valine (see Figure 2, and S19).