Antillatoxin: An Exceptionally Ichthyotoxic Cyclic Lipopeptide from the Tropical Cyanobacterium *Lyngbya majuscula*

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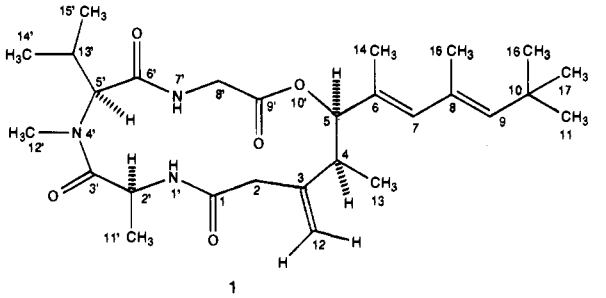
Marine cyanobacteria, particularly those collected from the wild, continue to be exceptionally rich sources of structurally-unique and biologically-active natural products. In this regard, we recently reported on our discovery and structural description of curacin A, a potent brine shrimp toxin from a marine cyanobacterium, *Lyngbya majuscula*, collected in Curaçao.[1] Curacin A shows considerable promise as an antiproliferative agent due to its inhibition of tubulin polymerization, a validated mechanism for treatment of neoplastic disorders.[2] However, in addition to brine shrimp toxicity, the crude extract of this *L. majuscula* collection showed considerable ichthyotoxic (LD_{50} approximately 25 μg/mL)[3] and molluskicidal (LD_{50} < 100 μg/mL)[4] activity. Curacin A was not responsible for these other bioactivities; rather, fractionation using the fish and snail bioassays has led to the isolation of two other distinct classes of natural products from this organism. The structure elucidation of the most ichthyotoxic compound, antillatoxin (**1**, LD_{50} = 0.05 μg/mL), is the subject of this report, whereas a strongly



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molluskicidal agent of novel structure, barbaramide A (LD_{100} = 10 μg/mL), is to be described in a future publication.[5] We speculate that this array of bioactive metabolites functions in nature to protect this cyanobacterium from predation by crustacea, herbivorous fish, and gastropod mollusks which are abundant in the marine habitat in which this organism thrives. Overall, the finding that the various bioactivities associated with the crude extract are due to three distinct classes of structurally-novel natural products further attests to the spectacular adaptive and biosynthetic capabilities of cyanobacteria, in general, and this collection of *L. majuscula* in particular.

Antillatoxin (**1**) was isolated in small yield as an amorphous powder (1.3 mg, 0.07% of extract) from the ichthyotoxic crude extract by repetitive chromatography on silica and RP-18 gels, followed by final purification using RP-18 HPLC.[6] At each stage the fish bioassay was used to monitor its isolation. Pure antillatoxin of $[\alpha]_D = -140°$ (*c* 0.13, MeOH) analyzed for

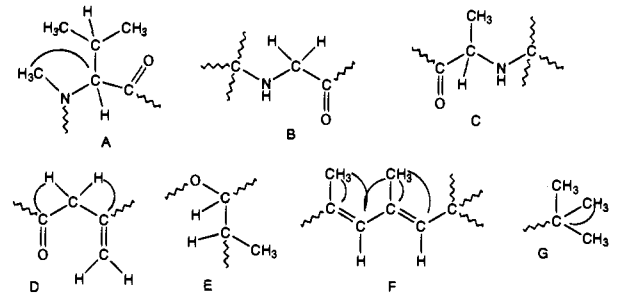

Figure 1. Partial structures A–G of antillatoxin (**1**) with diagnostic HMBC correlations.

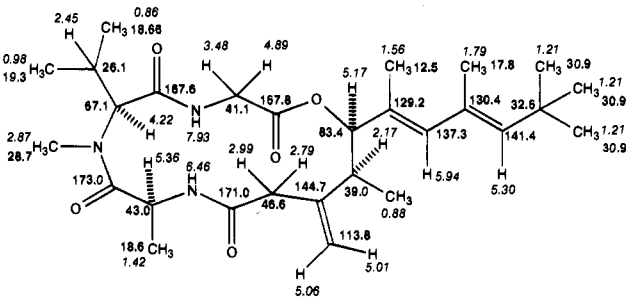

Figure 2. Complete structure of antillatoxin (**1**) with ^{1}H (italics) and ^{13}C (boldface) NMR assignments ($CDCl_3$, 400 MHz).

$C_{28}H_{45}N_3O_5$ by HR FABMS (2% TFA in 3-NBA, obsd $(M + H)^+$ *m/z* 504.3436, 0.2 ppm error for $C_{28}H_{46}N_3O_5$) and displayed strong amide carbonyl absorption typical of peptides ($\nu_{C=O}$ = 1639 cm^{-1}) and an ester carbonyl ($\nu_{C=O}$ = 1731 cm^{-1}).[7] Of the eight degrees of unsaturation inherent in this molecular formula, seven were defined from ^{13}C NMR data as four ester/amide carbonyls and six olefinic carbon atoms.[7] The two olefinic bonds were at least partially in conjugation (UV λ_{max} = 230 nm, ϵ = 12 000). Hence, antillatoxin was deduced to possess a single ring.

Analysis of antillatoxin by 1D and 2D ^{1}H and ^{13}C NMR allowed deduction of seven (A–G) partial structures which accounted for all of the atoms in the molecular formula. Three partial structures (A–C) were easily deduced as alanine, the methylene of glycine, and C2–C5 of valine. An *N*-methyl group at δ 2.87 was placed on the nitrogen of valine by long-range coupling (HMBC) between its carbon and the α-proton of valine (δ 4.22). Another spin system (D) was defined by COSY and LR COSY and consisted of an exocyclic olefin bordered on one side by a methylene group (δ 2.79 and 2.99). These chemical shifts were consistent with a methylene adjacent to both an olefin and a carbonyl, a hypothesis later substantiated by HMBC data (Figures 1 and 2). Partial structure E was composed of two adjacent methines, one of which was further coupled to a doublet methyl group; the other, H-5, had an associated carbon at a shift consistent with its attachment to a singly bound oxygen atom (δ 83.4). From long-range allylic coupling, HMBC data, and chemical shift information, the

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(6) The alcohol-preserved algal material (295 g dry weight) was repetitively extracted with 2:1 CH_2Cl_2/MeOH. The crude extract (3.3 g) was fractionated on silica gel with a gradient of hexane/EtOAc. The ichthyotoxic fraction was further fractionated on C18 cartridges with 80% MeOH. After confirming (^{1}H NMR, ichthyotoxicity) that the bioactive component was unreactive to CH_2N_2, the ichthyotoxic fraction was methylated and further purified by preparative TLC ($CHCl_3$/MeOH, 95:5). The final purification used HPLC on ODS using 80% MeOH and yielded 1.3 mg of antillatoxin.

(7) For ^{1}H and ^{13}C NMR data, see Figure 2. Coupling constants for antillatoxin: J_{2a-2b} = 12.7 Hz, J_{4-5} = 11.0 Hz, J_{4-13} = 7.0 Hz, J_{9-15} = 1.0 Hz, $J_{2'-3'}$ = 6.7 Hz, $J_{2'-11'}$ = 6.7 Hz, $J_{5'-13'}$ = 10.9 Hz, $J_{13'-14'}$ = 7.0 Hz, $J_{13'-15'}$ = 6.5 Hz, $J_{7'-8a'}$ = 1.5 Hz, $J_{7'-8b'}$ = 10.1 Hz, $J_{8a'-8b'}$ = 18.3 Hz. For additional analytical data, see supporting information (instruments as in Nagle and Gerwick: Nagle, D. G.; Gerwick, W. H. *J. Org. Chem.* **1994**, *59*, 7227–7237).

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conjugated diene deduced above from UV data was shown to possess two methyl groups and two protons. By coupling patterns as well as from NOESY data, these protons and methyl groups were each in a 1,3 relationship on the diene (partial structure F). The final partial structure (G) was observed in the ^{1}H NMR as a sharp singlet at δ 1.21 (9H), with associated carbons occurring as a 3C signal at δ 30.88. These data, in combination with a high-field quaternary carbon at δ 32.6, described a *tert*-butyl group.[8] Confirmation of this unsual arrangement was obtained from HMBC data which verified the coupling of this quaternary carbon to the 9H methyl singlet.

Connection of partial structures A–G was achieved through HMBC and NOESY spectra which were rich in correlations (Figure 1 and supporting information). Significantly, the tripeptide portion of antillatoxin was effectively sequenced as glycine–*N*-methylvaline–alanine through observation of α-CH, NH, and other nearby protons showing couplings to the three corresponding adjacent carbonyls. Two points of connection between the peptide and lipid portions of antillatoxin were shown through long-range C–X–H couplings (Ala NH proton to C1 of partial structure D, and H-2a to C1; ester carbonyl C9′ to Gly CH_2 and H-5 of partial structure E). Partial structures D and E were connected by long-range coupling between H-4 and the distinctive quaternary sp^2 carbon of the terminal olefin (C3). Connection between partial structure E and the conjugated diene (F) was possible from HMBC interactions between H-5 and C6 as well as a strong NOE between H-5 and H_3-14. Finally, two-bond couplings between C10 and both the H-9 vinyl proton and the *tert*-butyl group protons (partial structure G) completed the planar structure of antillatoxin.

The absolute stereochemistries of antillatoxin-derived *N*-methylvaline and alanine were established by acid hydrolysis of **1** (0.1 mg), HPLC separation of alanine and glycine from *N*-methylvaline, and stereochemical analysis by chiral phase TLC versus standards.[9] To the limits of detection, the antillatoxin-derived alanine and *N*-methylvaline were present only in the L configuration.

The stereochemistry at C4 and C5 was examined using a combination of molecular modeling, NOESY data, *J* values, and CD spectroscopy. Modeling of antillatoxin's structure was accomplished using a dynamic simulated annealing protocol[10] with the program XPLOR.[11] Four different structure files were defined within XPLOR corresponding to the 4*R*5*R*, 4*R*5*S*, 4*S*5*R*, and 4*S*5*S* configurations at C4 and C5 (Figure 3).[12] A total of 23 NOE and one torsion angle constraints were used in calculating the antillatoxin structure. Ten structure calculations were performed for each structure file. The NOE between the α protons of alanine and *N*-methylvaline could be accounted for by a *cis* peptide bond between these two residues. Inclusion of a hydrogen bond constraint between alanine NH and *N*-methylvaline carbonyl [indicated from the temperature dependent shift (3.4 ppb/deg) of this proton versus that of the NH of glycine (6.9 ppb/deg)] did not appreciably alter the calculated conformation, but did improve the rms deviation between structures. The calculated structures for the 4*R*5*S* configuration consistently violated NOE distance constraints or the torsion angle constraint, whereas the structures calculated for the other configurations satisfied the NOE and torsion angle constraints. The rms deviations between the heavy atoms in the 4*S*5*S*, 4*S*5*R*, and 4*R*5*R* structures were 2.5, 1.4, and 1.9 Å, respectively. We cannot unambiguously distinguish between these configurations

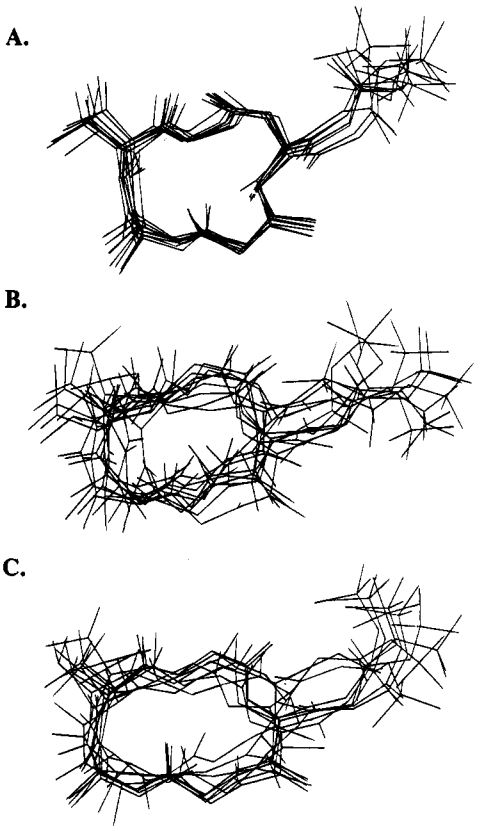

Figure 3. Three panels of superimposed energy-minimized structures for the NOE-permissible C4–C5 stereoisomers of antillatoxin (**1**): (A) 4*S*5*R*, assigned stereoconfiguration; (B) 4*R*5*R*; (C) 4*S*5*S*.

based solely on the modeling and NMR data. However, CD analysis of **1** showed a diene to olefin heterochromophoric exciton coupling at 227 nm, indicating a right-handed helicity between these groups and eliminating the 4*S*5*S* configuration. This result was best accounted for by the lowest energy conformation of the 4*S*5*R* stereoisomer, consistent with the modeling result suggested above. Hence, the complete absolute stereochemistry of antillatoxin (**1**) is here assigned as 4*S*, 5*R*, 2′*S*, 5′*S*.

Goldfish toxicity measurements with pure antillatoxin showed it to be among the most ichthyotoxic metabolites isolated to date from a marine plant (LD_{50} = 0.05 μg/mL, ED_{50} approximately 0.01 μg/mL)[3], and it is exceeded in potency only by the brevatoxins (BTX-A LD_{50} = 0.003 μg/mL against the freshwater zebra fish *Brachydanion rerio*).[13] Antillatoxin is a structurally novel lipopeptide, exceptional for its high degree of methylation, and without close parallel to any other known marine natural product.[14] The methyl substitution pattern in the lipid portion of **1** is consistent with a biogenesis principally from propionate subunits.[15]

Supporting Information Available: One-dimensional NMR (^{1}H, ^{13}C, and DEPT 135) and two-dimensional NMR (COSY, HMBC, NOESY), FABMS, IR, CD spectra, fish bioasssay and molecular modeling details (11 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(15) We gratefully acknowledge the permission and assistance in collection activities of the CARMABI Tropical Research Center in Curaçao. Molecular modeling (VH) was supported under a grant from the Medical Foundation of Oregon. Work in the College of Pharmacy was supported by the National Cancer Institute under Grant CA 52955.