Structure, Synthesis, and Biological Properties of Kalkitoxin, a Novel Neurotoxin from the Marine Cyanobacterium *Lyngbya majuscula*

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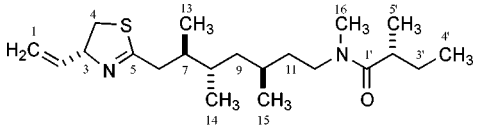
Cyanobacteria from a diversity of marine and freshwater habitats are known to produce neurotoxic secondary metabolites.[1] Herein, we describe the complete stereostructure, synthesis, and biological properties of kalkitoxin (**1**), a novel neurotoxic lipopeptide from a Caribbean collection of *Lyngbya majuscula*.

The organic extract of this *L. majuscula* exhibited potent brine shrimp and fish toxicity.[2] Using these assays, the toxic metabolite kalkitoxin (**1**), was isolated by sequential silica gel VLC, CC, and normal-phase HPLC (12.8 mg, 0.3% of extract). Subsequently, bioassay-guided fractionation using a primary cell culture of rat neurons in a microphysiometer[3] or inhibition of IL-1β stimulation of sPLA$_2$ in hepatocarcinoma cells[4] led to re-isolation of **1** in small yield from various Caribbean collections of *L. majuscula*.



Kalkitoxin (**1**)

Kalkitoxin (**1**) analyzed for $C_{21}H_{38}N_2OS$ indicated four degrees of unsaturation; from ^{13}C NMR analysis in DMSO-d_6 two were due to double bonds, one to a carbonyl group, and the remaining one to a ring system.[5] Data from E.COSY, HSQC, and a modified HSQMBC[6] experiments in benzene-d_6 allowed deduction of six partial structures for **1** (Supporting Information). One partial structure was composed of a *sec*-butyl group in which the methine component was deshielded to a chemical shift (δ 2.28) consistent with its being adjacent to a carbonyl. A second partial structure was composed of a methylated tertiary amide group which existed in two conformations (Supporting Information). A third partial structure possessed a deshielded methylene (δ 3.35) that could be sequentially connected by E.COSY to a second methylene group, and by HSQMBC to a methine and high-field methyl group. By E.COSY, an additional high-field methylene group (δ 1.10, 1.02) was adjacent to a methine which also bore a methyl group. The fifth partial structure was composed of a similar $-CH_2-CH-CH_3$ grouping; however, in this case, the methylene group protons were deshielded to δ2 .31 and δ 2.55. The final partial structure, based on E.COSY correlations, HSQMBC, and chemical shift models,[7] was composed of a thiazoline ring with an ethylene appendage; this was further substantiated by EIMS fragmentations (Supporting Information). HSQMBC data were used to connect these partial structures and gave the full planar structural assignment of **1**.

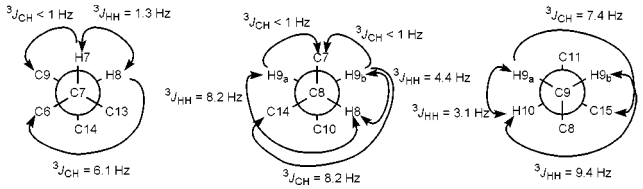


Figure 1. Representation of rotamers about C7, C8, C9, and C10 with depiction of all heteronuclear and homonuclear couplings that were used to define the relative stereochemistry at C7, C8, and C10 using the *J*-based configuration approach.[8]

The C3 stereochemistry of kalkitoxin was determined by Marfey's analysis. Kalkitoxin was ozonized and then hydrolyzed in 6 N HCl to obtain cysteic acid. Marfey's analysis of this hydrolysate yielded L-cysteic acid, defining C3 as *R*. The limited amount of kalkitoxin precluded determination of the C2′ stereochemistry. The relative stereochemistry of the three chiral centers within the aliphatic chain of kalkitoxin (C7, C8, C10) was determined using the *J*-based configuration analysis method.[8]

The $^3J_{CH}$ values were measured by a modification of the recently reported HSQMBC pulse sequence,[6] and the $^3J_{HH}$ values were determined utilizing the E.COSY pulse sequence.[9] To overcome the limited sample size of natural kalkitoxin (~300 μg of **1** remained because of chemical instability) all data used in this analysis were recorded on a Bruker 500 MHz DRX spectrometer equipped with a Bruker 5 mm TXI CryoProbe (benzene-d_6). The relative stereochemistry at C7−C8 was suggested by observation of a small (1.3 Hz gauche) $^3J_{HH}$ between H7−H8, a large (6.1 Hz, anti) $^3J_{CH}$ between H8−C6, and a small (<1 Hz, gauche) $^3J_{CH}$ between H7−C9 (see Figure 1). Stereochemistry at positions C8 and C10 were related through the intervening C9 diastereotopic methylene protons. The low-field proton at C9 (H9$_a$) showed a large (8.2 Hz, anti) $^3J_{HH}$ to H8,

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(2) This brownish-colored *L. majuscula* was collected from 6 to 8 m water depth (1 L compressed volume), 11 August, 1994, Playa Kalki, Curaçao (Collection Code NAK-11Aug94-03); preserved at reduced temperature in isopropyl alcohol until extraction; 4.32 g organic extract.

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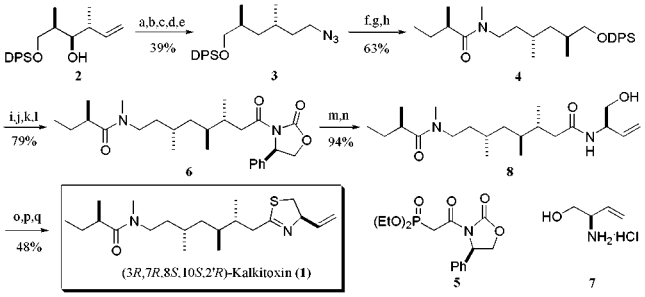
Scheme 1. Synthesis of (3*R*,7*R*,8*S*,10*S*,2′*R*)-Kalkitoxin (**1**)[a]



[a] (a) *n*-BuLi, THF; *p*TsCl. (b) $LiAlH_4$, THF reflux. (c) 9-BBN, ultrasound, THF; aq NaOH, H_2O_2. (d) MsCl, Et_3N, DMAP, CH_2Cl_2. (e) NaN_3, DMF, 55 °C. (f) H_2 (1 atm) 5% Pd−C, EtOAc. (g) (*R*)-2-methylbutyric acid, DEPC, Et_3N, DMF. (h) *n*-BuLi, THF; MeI. (i) TBAF, THF. (j) Py·SO_3, Et_3N, DMSO, CH_2Cl_2. (k) Phosphonate **5**, NaHMDS, THF. (l) MeMgBr, CuBr-DMS, THF/DMS, −30 °C. (m) aq LiOH, H_2O_2, THF. (n) amino alcohol **7**, EDCI·HCl, *i*Pr_2NEt, DMAP, CH_2Cl_2. (o) DAST, CH_2Cl_2, −20 °C. (p) H_2S, Et_3N, MeOH. (q) DAST, CH_2Cl_2, −20 °C.

whereas the high-field proton ($H9_b$) showed a small (4.4 Hz, gauche) $^3J_{HH}$ to H8. Additionally, small (<1 Hz) $^3J_{CH}$ were observed from $H9_a$ and $H9_b$ to C7, and a large (8.2 Hz, anti) $^3J_{CH}$ from $H9_b$ to C14. The relative stereochemistry at C9 and C10 was determined by a large (9.4 Hz, anti) $^3J_{HH}$ between $H9_b$ and H10 and a small (3.1 Hz, gauche) $^3J_{HH}$ for $H9_a$−H10. Finally, a large (7.4 Hz, anti) $^3J_{CH}$ was measured for $H9_a$−C15. In summary, these data strongly supported a 7*R**, 8*S**, 10*S** relative stereochemistry for **1**. In combination with the above-determined 3*R* absolute stereochemistry, the total number of stereochemical possibilities was reduced to four.

To determine the absolute stereochemistry of natural kalkitoxin, kalkitoxins having all possible configurations were synthesized; (3*R*,7*R*,8*S*,10*S*,2′*R*)-kalkitoxin was found to be identical with the natural substance **1**. The chemical synthesis of natural **1** commenced with the known alcohol **2**.[10a] Deoxygenation of the corresponding tosylate, hydroboration, and conversion of the resulting alcohol into the azide gave **3** in 39% overall yield from **2**.[10b−d] Reduction of the azide, coupling with (*R*)-2-methylbutyric acid using DEPC,[10e] and *N*-methylation gave *N*-methylamide **4** in 63% yield. *O*-Desilylation and oxidation was followed by a Horner-Emmons reaction and simultaneously homologated and introduced the (*R*)-phenylglycine-derived auxiliary required for an asymmetric conjugate addition.[10f] Introduction of the C7 methyl group by the method of Hruby[10g] proceeded smoothly to give a methyl adduct **6** as a single isomer in 79% overall yield from **4**. Removal of the chiral auxiliary and coupling with (*R*)-amino alcohol **7**[10h] yielded **8** in 94% yield. Following Wipf's oxazoline−thiazoline interconversion protocol,[10i] cyclodehydration of the amide with DAST to the oxazoline[10j] and then treatment with hydrogen sulfide afforded the thioamide. Finally, a second cyclodehydration with DAST[10k] provided (3*R*,7*R*,8*S*,10*S*,2′*R*)-kalkitoxin (**1**) in 48% overall from the amide **8** (Scheme 1).[11]

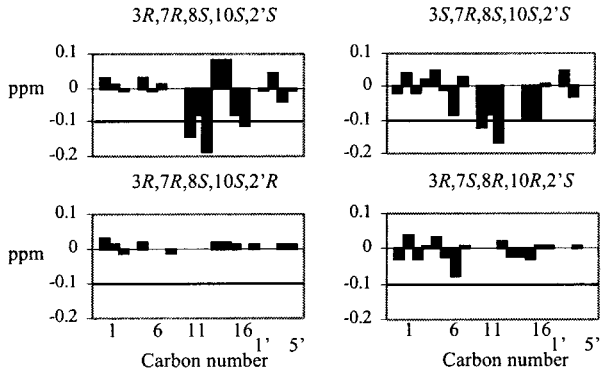


Figure 2. Differences in ^{13}C NMR shifts between natural kalkitoxin (**1**) and four synthetic kalkitoxin stereoisomers.

Comparison of ^{13}C NMR chemical shifts between five synthesized diastereoisomers and natural kalkitoxin showed very small differences of less than 0.2 ppm (Figure 2). However, both the 3*S*,7*S*,8*R*,10*R*,2′*S*- and 3*R*,7*R*,8*S*,10*S*,2′*R*-isomers showed maximal ^{13}C NMR differences of 0.026 ppm. The CD spectrum of the 3*S*,7*S*,8*R*,10*R*,2′*S*-isomer was of equal intensity but opposite sign to natural kalkitoxin. Correspondingly, the CD of the 3*R*,7*R*,8*S*,10*S*,2′*R*-isomer was essentially identical to natural compound **1** (Supporting Information).

Natural (+)-kalkitoxin (**1**) was strongly ichthyotoxic to the common goldfish (*Carassius auratus*, LC_{50} 700 nM), potently brine shrimp toxic (*Artemia salina*, LC_{50} 170 nM), and potently inhibited cell division in a fertilized sea urchin embryo assay (IC_{50} ~25 nM).[12a] Synthetic (+)-kalkitoxin (3*R*,7*R*,8*S*,10*S*,2′*R*) was equally potent in the brine shrimp assay (LC_{50} 170 nM). Interestingly, synthetic (−)-kalkitoxin (3*S*,7*S*,8*R*,10*R*,2′*S*) was relatively inactive as a brine shrimp toxin (LC_{50} 9300 nM). In a primary cell culture of rat neurons, natural kalkitoxin displayed an exceptional level of neurotoxicity (LC_{50} 3.86 nM), and its effects were inhibitable with NMDA receptor antagonists.[1c] Additionally, natural kalkitoxin is highly active in an inflammatory disease model which measures IL-1β-induced $sPLA_2$ secretion from HepG2 cells (IC_{50} 27 nM).[4] Finally, preliminary evidence suggests that kalkitoxin is an exquisitely potent blocker of the voltage sensitive Na^+ channel in mouse neuro-2a cells (EC_{50} of **1** = 1 nM; EC_{50} of saxitoxin = 8 nM).[12b] Consistent with many known cyanobacterial metabolites, kalkitoxin appears to derive from a mixed polyketide/nonribosomyl peptide synthetase pathway.[13]

Supporting Information Available: Experimental details and NMR and CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Information on the syntheses of kalkitoxin and its stereoisomers is found in part in the Supporting Information and will be presented in detail elsewhere.

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