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Structure and Absolute Configuration of Karlotoxin-2, an Ichthyotoxin from the Marine Dinoflagellate *Karlodinium veneficum*

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Abstract

In an attempt to determine the cause of repeated fish kills in an estuarine aquaculture facility in Maryland, a toxin was isolated from *Karlodinium veneficum* with hemolytic, cytotoxic, and ichthyotoxic properties, which was referred to as karlotoxin-2 (KmTx2). The structure of KmTx2 was elucidated by means of detailed 1D and 2D NMR spectra including 2D INADEQUATE. The relative and absolute configuration of karlotoxin-2 was determined using *J*-based configuration analysis and comparison of degradation products with synthetic controls.

An increase in the frequency of ocean disease events over the past two decades with significant implications on marine life has contributed to rising awareness of environmental and human health issues in marine/estuarine environments and the need for rigorous scientific approaches to address them.¹ On July 30, 1996 a large mortality of ca. 15,000, 1–1.25 lb. hybrid striped bass occurred during a dinoflagellate bloom at Hyrock Fish Farm Maryland. After that, many

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Supporting Information **Available:** Detailed experiments, configuration analysis, and biological activity are presented. NMR data assignment and spectra for karlotoxin-2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

massive fish kill events have been attributed to this organism. These events were unusual, because human health effects among individuals exposed to the presumably contaminated waters were reported, leading to descriptions of a new toxic exposure syndrome characterized by unique skin lesions, respiratory problems, and neurological complications primarily including short-term memory loss.² These have resulted in substantial economic losses in the local seafood and tourism industries. These events have in the past been attributed to the dinoflagellate *Pfiesteria piscicida*³ but controversial results have been reported about *Pfiesteria* toxins.⁴ Several structural classes have been proposed for the *Pfiesteria* toxin and a highly labile copper containing organo-metallic complex has been proposed as the putative toxin in a recent report.⁵ A definitive link between *Pfiesteria piscicida* and these events has yet to be conclusively determined after thirteen years and countless fish kills.

In an attempt to determine the cause of repeated '*Pfiesteria*-associated' fish kills in Maryland, a co-occurring dinoflagellate *Karlodinium veneficum* was identified and was subsequently shown to produce a unique suite of water soluble toxins with hemolytic, cytotoxic, and ichthyotoxic properties and are referred to as karlotoxins (KmTx).⁶ To date these toxins have been detected *in situ* and in clonal isolates collected from estuarine waters from the U.S. states of Maryland, North and South Carolina, Georgia, Florida, Mississippi as well as isolates from New Zealand, Norway, and the English Channel. In addition, these same toxins were isolated directly from water samples collected during fish kills in a South Carolina brackish water pond,⁷ at fish kills in a tributary of the Chesapeake Bay and the Swan River in Perth, Australia.⁸ In all cases high densities of *K. veneficum* were found regardless of the presence of *Pfiesteria piscicida*. The principal toxin isolated from both cultured cells and directly from water samples collected during the fish kills (KmTx2), was similar but not identical to the karlotoxin isolated from cultures and fish kills in Maryland (KmTx1).⁹

Karlotoxin-2 was obtained from a clonal culture of *Karlodinium veneficum* (CCMP 2064) collected from a fish kill in Georgia. The molecular formula $C_{67}H_{121}ClO_{24}$ was deduced from detailed analysis of NMR data and the high resolution ESI-TOFMS molecular ion series $1367.7844 [M+Na]^+$ (calcd. 1367.7829), $695.3883 [M+2Na]^{++}$ (calcd. 695.3866), with careful comparison with simulated isotope pattern data for this formula. Partial structures were assigned based on the preliminary NMR spectral data obtained from a limited quantity of unenriched material (<1 mg), which clearly revealed a polyketide. A ^{13}C enriched karlotoxin-2 (1.3 mg) was prepared from 40 L of culture with $NaH^{13}CO_3$ (50 mg/L). From this ^{13}C uniformly enriched sample (10% ^{13}C), high quality ^{13}C , DEPT, HMQC, HMBC, and 2D INADEQUATE spectra were obtained using a 600 MHz NMR instrument equipped with a dual CryoProbe. The carbon backbone connection of C1~C22, C23~C33, C34~C39, C40~C53, and C58~C63 were established from the 2D INADEQUATE spectrum and were verified by COSY, TOCSY, HSQC and HMBC. A weak INADEQUATE correlation between C-30 and C-31 was validated by the COSY correlations between H-30 at δ 3.91 and H-31 at δ 1.84 and 1.70. The connection of C-22 and C-23 was confirmed by the HMBC correlations of H-23 to C-22, C-21, and H-22 to C-23, C-24, and by the COSY correlation between H-22 at δ 3.69 and H-23 at δ 3.33. The connection of C-39 and C-40 was deduced from the correlations of H-67 at δ 5.08 and 5.03, H-41 at δ 4.23 to C-39 at δ 27.56, H-39 at δ 2.45, 2.15,

H-38 at δ 1.70 to C-40 at δ 151.40. Although the resonances of H-33 and H-34 are overlapped at δ 3.73, and the resonances of C-33 and C-34 at δ 73.17 and 73.18 are too close to be distinguished in 2D NMR, the connection of C-33 and C-34 was established by the HMBC correlation between δ 3.73 and δ 73.17/73.18, which can only be reasonably explained as the correlation between H-33 and C-34, and H-34 and C-33. A $(\text{CH}_2)_4$ moiety was deduced from the DEPT, HMQC, molecular formula, and ^{13}C shift values, which is the link between C-53 and C-58. The ether bridges between C-43 and C-47, and C-32 and C-36 were determined by the HMBC correlations of H-47 at δ 3.74 to C-43 at δ 70.40 and H-36 at δ 3.40 to C-32 at δ 74.72.¹⁰

The relative configuration of the two pyran rings was determined using chemical shifts, coupling constants and NOE interactions. The relative configuration of substructures C14-C18, C21-C24, C28-C37, and C41-C49 were established using *J*-based configuration analysis¹¹, while the relative configuration of the isolated chiral centers at C-2, C-6, C-10 could not be resolved by this technique. This method was reported by Murata et al. for the analysis of the relative configuration of polyhydroxylated acyclic molecules by detailed analysis the $^3J_{\text{H,H}}$ and $^2,^3J_{\text{C,H}}$ values, and has been successfully applied to several examples,¹² including the determination of the relative configuration of amphidinol 3,¹³ with which karlatoxin-2 shares significant structural similarities. $^3J_{\text{H,H}}$ values were determined by 1D TOCSY and homonuclear decoupling experiments, and $^2,^3J_{\text{C,H}}$ values by sensitivity- and gradient-enhanced hetero (ω_1) half-filtered TOCSY (HETLOC)¹⁴ or 2D gradient-selected HSQMBC15. For example, the relative configuration of C14~C16 was determined through the assignment of the two diastereomeric methylene protons of C15 (Figure 1). The anti-orientations of H15(H)/H16 and H15(L)/H14 were suggested by the large $^3J(\text{H15H}, \text{H16})$ and $^3J(\text{H15L}, \text{H14})$, while gauche orientations of H15(L)/H16 and H15(H)/H14 were provided by the small $^3J(\text{H15L}, \text{H16})$ and $^3J(\text{H15H}, \text{H14})$. The anti-orientation of H15L/C64 and H15H/C14-OH were shown by large $^3J(\text{H15L}, \text{C64})$ and small $^2J(\text{H15H}, \text{C14})$. These interactions established the relative configuration of C14~C16 as depicted in Figure 1a. The intermediate coupling constants between H18/H17 and H17/H16 suggested two alternative conformations for both C18/C17 and C17/C16. The small $^3J(\text{H17}, \text{C19})$ and $^3J(\text{H17}, \text{C64})$ indicated that both H17/C19 and H17/C64 kept in gauche orientation. The $^2J(\text{H17}, \text{C18})$, $^2J(\text{H18}, \text{C17})$, and $^2J(\text{H16}, \text{C17})$ showed intermediate between gauche and anti orientation. Thus, the configuration of C16~C18 was determined as shown in Figure 1b. The relative configurations of C21~C24, C28~C32, C36~C37, C41~C43, and C47~C49 were elucidated using the same method (see supporting information).

The relative configuration of C37-C41 was correlated using coupling constants and NOE data as shown in see Figure 2. Thus, the configuration of three subunits, e.g. C14~C18, C21~C24, and C28~49, were established.

To determine the absolute configuration, KmTx2 was first degraded using HIO_4 , then reduced by NaBH_4 , followed by esterification with (*R*)- and (*S*)- α -methoxyphenylacetic acid (MPA). The MPA esters of degradation products corresponding to C2~C17 (**2b/c**), C18~C22 (**3b/c**), and C24~C29 (**4b/c**) were obtained (see Figure 3). The absolute configurations of C-6, C-10, C-14, C-21, and C-28 were assigned as *R*, *R*, *S*, *S*, *R*, respectively, by comparison of $\Delta\delta^{\text{RS}}$ between **2b/2c**, **3b/3c**, and **4b/4c** (Figure 3). To verify the absolute configurations of C-21 and C-28, **3b/3c** and **4b/4c** were synthesized from dimethyl (*S*)-2-methylglutarate and (*R*)-3-methyl-cyclohexanone.¹⁶ The NMR data of the degradation products **3b/3c** and **4b/4c** match with the synthesized **3b/3c** and **4b/4c** respectively, which confirmed the determination of 21-*S* and 28-*R* configuration. The absolute configuration of C16~C18, C20~C22 and C27~C49 were correlated with C-14, C-21, and C-28, respectively as shown in Figure 3. To determine the absolute configuration of C-2, karlatoxin-2 was degraded through olefin metathesis using Grubbs' catalyst II and excess of ethylene to yield pent-4-ene-1,2-diol (**5a** or **5b**). The

degradation product exhibited the same retention time in chiral GC/MS with the synthetic (*R*)-enantiomer (**5a**), indicating a *R* configuration for C-2 (Figure 3). Karlotoxin-2 shares structural similarities to amphidinol 3,¹³ while the absolute configuration of C32-C49 unit is opposite to that of amphidinol 3. The differences in absolute configuration could be due to limited coupling constant data available between C28-C32 of KmTx2 or similar challenges with amphidinol 3. Amphidinol 3 shares the same C32~C49 substructure with KmTx2 less a 35-OH and with addition of 31-OH group. The absolute configuration of C32~C49 of amphidinol 3 was determined based on correlations with C-37, whose absolute configuration was deduced from Mosher's esters of the C31~C48 unit of the HIO₄ degradation products.¹³ The crowded hydroxyl groups (31, 33, 34, and 37) and as a result the crowded MTPA esters in this degradation subunit (C31~C48), may affect a correct prediction of the absolute configuration of C-37 of amphidinol 3 (see supporting information for details).

The purified KmTx2 reproducibly kills fish in a dose dependent manner. The mortalities in zebrafish and sheepshead minnow juveniles are observed at 0.1~0.5 µg/mL, which is within the concentrations of KmTx2 (0.1~0.8 µg/mL) in water samples during fish kills.⁴ The pathology (sloughing of epithelium) observed in the laboratory treated fish gills (Fig 4B) are similar to that observed in fish kills in nature (See Figure 4C). Karlotoxins have been shown to be membrane, sterol specific toxins, which is similar to amphotericin B and amphidinol 3.¹⁷ The sterol binding is greatest for brassicasterol > cholesterol > ergosterol > gymnodinosterol (the dominant sterol found in *K. veneficum*).

K. veneficum, formerly *Gyrodinium galatheanum* or *Gymnodinium veneficum*, is distributed worldwide¹⁸ and has been reported to produce toxins since first isolated at the Plymouth Marine Laboratory in the UK by Mary Parke during the 1950's,¹⁹ however, the complete structure of this algal toxin class has yet to be revealed until this report. Karlotoxin is an intracellular compound and greater than 90% of karlotoxin is cell-associated. However, it is easily released by shear forces like centrifugation, filtration or passage across gill rackers in feeding menhaden.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a. C14~C16

$^3J_{(H-14, H-15H)}$	3.3
$^3J_{(H-14, H-15L)}$	9.7
$^2J_{(C-14, H-15H)}$	-1.6
$^2J_{(C-14, H-15L)}$	-5.4

$^3J_{(H-16, H-15H)}$	10.7
$^3J_{(H-16, H-15L)}$	3.3
$^3J_{(C-64, H-15H)}$	3.1
$^3J_{(C-64, H-15L)}$	6.2

b. C16~C18

$^3J_{(H-18, H-17)}$	4.2
$^3J_{(C-19, H-17)}$	small*
$^2J_{(C-18, H-17)}$	-1.4
$^2J_{(C-17, H-18)}$	-1.8

$^3J_{(H-17, H-16)}$	6.3
$^3J_{(C-64, H-17)}$	1.5
$^2J_{(C-17, H-16)}$	-3.0

Figure 1.
Rotamers and coupling constants for C14~C18

Figure 2.
Correlation of relative configuration of C37~C42 and coupling constant and NOE data

Figure 3.
Degradation followed by esterification with MPA and $\Delta\delta^{RS}$ values

Figure 4. Histological sections (5-6 μm thickness) of zebrafish (*Danio rerio*) juveniles (*Danio rerio*) stained with hematoxylin and eosin (H&E) after exposure. (A) Control gill arch. (B) Karlotoxin-2 exposure (0.5 $\mu\text{g}/\text{ml}$ for 1 hr) Note loss of intra-lamellar spacing and secondary lamellar fusion. (C) Gut epithelia of a striped killifish (*Fundulus majalis*) exposed in situ (~ 0.2 $\mu\text{g}/\text{ml}$) during a fish kill event to karlotoxin.