

Structure and Relative Potency of Several Karlotoxins from *Karlodinium veneficum*

Ryan M. Van Wagoner,[†] Jonathan R. Deeds,[‡] Avery O. Tatters,[†] Allen R. Place,[§] Carmelo R. Tomas,[†] and Jeffrey L. C. Wright^{*,†}

Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin Moss Lane, Wilmington, North Carolina 28409, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, Maryland 20740, and Institute for Marine and Environmental Technology, University of Maryland Center of Environmental Sciences, 701 East Pratt Street, Suite 236, Baltimore, Maryland 21202

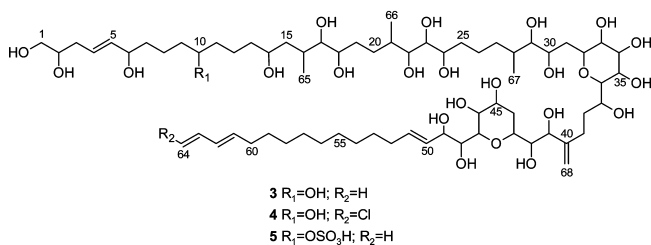
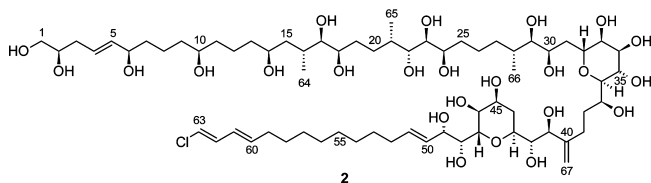
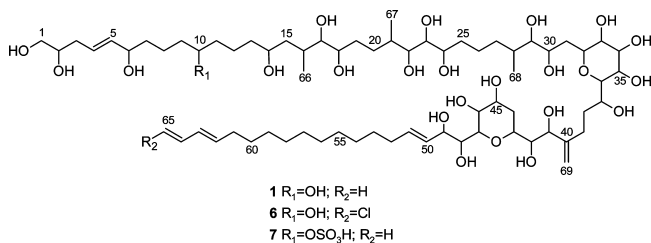
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The karlotoxins are a family of amphidinol-like compounds that play roles in avoiding predation and in prey capture for the toxic dinoflagellate *Karlodinium veneficum*. The first member of the toxin group to be reported was KmTx 1 (**1**), and here we report an additional five new members of this family (**3–7**) from the same strain. Of these additional compounds, KmTx 3 (**3**) differs from KmTx 1 (**1**) in having one less methylene group in the saturated portion of its lipophilic arm. In addition, 64-*E*-chloro-KmTx 3 (**4**) and 10-*O*-sulfo-KmTx 3 (**5**) were identified. Likewise, 65-*E*-chloro-KmTx 1 (**6**) and 10-*O*-sulfo-KmTx 1 (**7**) were also isolated. Comparison of the hemolytic activities of the newly isolated compounds to that of KmTx 1 shows that potency correlates positively with the length of the lipophilic arm and is disrupted by sulfonation of the polyol arm.

The karlotoxins are a class of amphidinol-like compounds produced by mixotrophic strains of the dinoflagellate *Karlodinium veneficum*.^{1,2} The karlotoxins have been reported to display a variety of interesting effects on biological systems including cellular lysis,^{1,3–6} damage of fish gills,^{1,3,7–9} and immobilization of prey organisms.¹⁰ There is growing evidence that the karlotoxins support a number of ecological roles for *K. veneficum* including deterring predation¹¹ and assisting prey capture.^{2,12} The cytolytic activity of the karlotoxins is modulated by membrane sterol composition, which has been proposed as a mechanism for *K. veneficum* avoiding autotoxicity.^{13–15} *K. veneficum* has been implicated in several fish kill events apparently caused by the damaging effects of the karlotoxins.^{1,3,12,16,17}

Two families of karlotoxins have been described as belonging to the KmTx 1 and KmTx 2 groups, which differ from one another in UV absorbance maxima, potency, and geographic distribution.¹⁸ Although the reports of toxic compounds from *K. veneficum* (originally *Gymnodinium veneficum*) date back to the 1950s,⁷ it has only been in recent years that structures were reported for KmTx 1 (**1**)⁴ and KmTx 2 (**2**),^{17,19} including the absolute configuration for the latter compound.¹⁹ With the structures of KmTx 1 (**1**) and KmTx 2 (**2**) now reported, the difference between the two compounds in carbon chain structure is localized to the length of the lipophilic side chain. In KmTx 1 (**1**) the side chain is 18 carbons in length (C-48–C-65), whereas in KmTx 2 (**2**) it is two carbons shorter (C-48–C-63). The karlotoxins, like the amphidinols produced by dinoflagellates of the genus *Amphidinium*,²⁰ have a hairpin-like structure with three distinct regions: a polyol arm that exhibits variable hydroxylation and methylation; a hinge region containing two ether rings; and a lipophilic arm that often includes conjugated trienes in amphidinols but instead in karlotoxins contains a terminal diene that gives these compounds their distinctive UV spectra. In order to better delineate the structural basis for differing hemolytic potencies among the karlotoxins, we undertook the isolation and structure determination of five new congeners and determined their hemolytic activity. The resulting structures show that, contrary to previous observations,^{5,6,18,21} it is possible for KmTx 1-like and KmTx 2-like compounds to occur in the same

organism. Moreover, our results indicate that the length of the lipophilic arm is an important determinant for potency and that sulfonation might provide an effective means of reducing toxicity of the karlotoxins.



Results and Discussion

Cultures of *K. veneficum* were harvested by filtration, and the supernatant was subjected to solid-phase extraction, following previous observations of the release of karlotoxins upon filtration.¹ The recovered desalted material was subjected to C₁₈ flash chromatography using a step gradient, leading to elution of polar karlotoxins around 60–80% MeOH and less polar karlotoxins around 80–100% MeOH. The latter fraction was subjected to C₁₈ HPLC to yield **3**, **4**, and **6** in addition to KmTx 1.⁴ The more polar fraction was fractionated by C₁ HPLC to yield **5** and **7**.

* To whom correspondence should be addressed. Phone: 910 962 2397. Fax: 910 962 2410. E-mail: wrightj@uncw.edu.

[†] UNC Wilmington.

[‡] USFDA.

[§] University of Maryland Center of Environmental Sciences.

High-resolution mass spectrometry of purified KmTx 3 (**3**) suggested a molecular formula of $C_{68}H_{124}O_{24}$ ($\Delta = -2.0$ ppm), which differs from that of KmTx 1 (**1**) by a loss of CH_2 .⁴ Comparison of the 1D and 2D NMR spectra of **3** and KmTx 1 (**1**) showed them to be nearly identical to each other. The molecular formula was supported by the 1H and ^{13}C NMR spectra, the latter of which revealed 68 signals, which were assigned to one quaternary sp^2 carbon, 7 sp^2 methines, two sp^2 methylenes, 25 oxygenated methines, one oxygenated methylene, three aliphatic methines, 26 aliphatic methylenes, and three aliphatic methyls (Table 1). This differs from a similar count for KmTx 1 (**1**) by one aliphatic methylene. All of the distinct aliphatic methylene signals for **1** could be accounted for in the 1H NMR spectrum except those in the saturated chain from C-53 to C-59.⁴ Thus, the combined data suggested the terminal diene and vinyl group of the lipophilic arm in **3** are bridged by nine methylenes rather than the 10 occurring in **1**. Such a structure is also consistent with the observation of seven distinct signals in the range δ_C 30.1–30.6 ppm, accounting for C-53–C-59 (Table 1).

Further analysis of the 2D NMR data including HSQC-TOCSY, HMBC, and ROESY indicated that all of the structural features of **1** are also present in **3**, including the two ether rings. Because the 1H NMR data for **3** were acquired at 500 MHz and those for **1** at 800 MHz, there were more instances of overlapped resonances in **3**. Nevertheless, for the signals in **3** that were resolved and from which coupling patterns could be measured, all had nearly identical coupling constants with those reported in **1**. In addition, the same cross-annular ROESY cross-peaks observed in KmTx 1 (**1**)⁴ were also found in the spectra for KmTx 3 (**3**), indicating that the two compounds likely have identical configurations for all stereocenters.

Further support for structure **3** was provided by comparison of the mass spectrum resulting from collisionally induced dissociation (CID) of KmTx 3 (**3**) to that of KmTx 1 (**1**).⁴ Most of the fragment masses were the same between the two compounds, with the main differences occurring for m/z 433 and 1287, both of which are 14 Da lower than the corresponding fragments in KmTx 1. Both differences can be explained using the fragmentation pathways originally described for KmTx 1 (Figure 1).⁴ Thus, **3** is intermediate in carbon chain length between KmTx 1 (**1**) and KmTx 2 (**2**) and represents a third group within the karlotoxin family.

The high-resolution mass spectrum of **4** suggested a molecular formula of $C_{68}H_{123}ClO_{24}$ ($\Delta = -0.4$ ppm). A prominent second isotope peak was observed, consistent with the presence of chlorine. The difference in formulas between **3** and **4** suggests the replacement of a hydrogen atom with a chlorine atom. Comparison of the CID MS-MS spectra of **3** and **4** further suggested that the location of the chlorine atom is within the lipophilic arm (Figure 1). In particular, the occurrence of a shift to higher mass by 34 Da in the fragmentation product resulting from cleavage of the bond C-41/C-42 (m/z 467 in **4** vs m/z 433 in **3**) and the presence of a common fragment at m/z 1111 resulting from cleavage of the bond C-48/C-49 were crucial in locating the chlorine atom (Figure 1). In addition, the λ_{max} in the UV spectrum of **4** shifted to 238 nm compared to **3**, which had a λ_{max} of 228 nm, which further suggested that the chlorine atom was attached to the terminal diene of the lipophilic arm. This shift in UV absorbance has been noted as a distinguishing feature between KmTx 2 (which bears a chlorine atom on its terminal olefinic carbon)¹⁹ and KmTx 1, suggesting that **4** likewise has a chlorinated diene. Comparison of the 2D NMR spectra of **3** and **4** identified the terminal diene as being the attachment site for the chlorine atom and that C-64 in **4** is a methine rather than a methylene (Table 1). The large coupling constant for H-64 (13.0 Hz) indicates that the terminal alkene in **4** has an *E* substitution pattern, thus identifying **4** as 64-*E*-chloro-KmTx 3.

The molecular formula of **5** was determined by high-resolution mass spectrometry as $C_{68}H_{124}O_{27}S$ ($\Delta = -1.5$ ppm) and differed from that of **3** by SO_3 , suggesting the presence of a sulfate group.

Such a difference is consistent with the earlier elution of **5** from a reversed-phase column compared with **3**. Comparison of the HSQC spectra of **3** and **5** revealed the only difference to be in the oxymethine region with the disappearance of the 3.56 ppm/72.0 ppm correlation (C-10; Table 1) and the appearance of a new correlation at 4.49 ppm/79.9 ppm (Table 1), thus identifying the hydroxyl group at C-10 as the site of attachment of the sulfonate group. Further confirmation of this assignment was provided by TOCSY correlations between H-10 and both H-6 and H-14, both of which were identical in δ_H and δ_C to their counterparts in **3** (Table 1). The only other notable differences in NMR data were the overlapping shifts of the flanking positions 9 and 11, now both shifted to δ_H 1.69, δ_C 34.9 ppm (Table 1). Together, the data identify **5** as being 10-*O*-sulfo-KmTx 3. Although negative-ion MS-MS of sulfate-bearing amphidinols often yields highly informative spectra due to charge-remote fragmentation,^{20,22–25} attempts at CID by negative-ion ESIMS-MS at various collision energies yielded only varying ratios of precursor ion and m/z 97 (OSO_3^-), perhaps due to the attachment of the sulfonate group on an internal secondary alcohol.

The molecular formulas for **6** ($C_{69}H_{126}ClO_{24}$; $\Delta = +1.2$ ppm) and **7** ($C_{69}H_{127}O_{27}S$; $\Delta = -2.6$ ppm) suggested that these two compounds bear the same relationship to KmTx 1 (**1**) that **4** and **5** (respectively) do to KmTx 3 (**3**). For example, comparison of the NMR spectra of **6** with those of **4** showed them to be nearly identical in all respects (Tables 1 and 2). Moreover, the fragment ions at m/z 467 and 481 for **4** and **6**, respectively, are consistent with a difference of one methylene in the lipophilic side chain, while the fragment ion at m/z 1111 in **3**, **4**, **6**, and **1** is also consistent with the location of the chlorine atom in this lipophilic chain (Figure 1). Thus the combined data identified **6** as 65-*E*-chloro-KmTx 1. Similarly, the absence of observable differences in NMR shifts for **7** compared to **5** (Tables 1 and 2) identified **7** as 10-*O*-sulfo-KmTx 1.

The hemolytic potencies of **3**–**7** were determined to provide a greater understanding of structure–activity relationships within the karlotoxins. The results of the assays with human erythrocytes are given in Table 3. The amounts of toxin isolated were low, so the results should be interpreted with caution. However, there do appear to be consistent results in comparing congeners of KmTx 1 with those of KmTx 3. The results indicate that the length of the lipophilic arm has a considerable effect on potency. KmTx 3 (**3**) is approximately 3-fold less potent than KmTx 1 (**1**; EC_{50} of 63 nM).⁴ The chlorinated congener 64-*E*-chloro-KmTx 3 (**4**) is approximately twice as potent as **3** but half as potent as 65-*E*-chloro-KmTx 1 (**6**), which contains a slightly longer lipophilic side chain. However, the relative increase in potency observed in comparing 64-*E*-chloro KmTx 3 (**4**) to KmTx 3 (**3**) is considerably higher than that seen for 65-*E*-chloro KmTx 1 (**6**) vs KmTx 1 (**1**) (Table 3). Similarly, the loss in potency upon sulfonation of KmTx 3 (12-fold for **5**; Table 3) was much more pronounced than that seen for **7** compared to KmTx 1 (5-fold; Table 3). Overall, this suggests the existence of a binding penalty for lipophilic chains shorter than that of KmTx 1 (**1**) that can be somewhat ameliorated by extension of the lipophilic arm with chlorine, but is exacerbated by sulfonation of the polyol arm.

Before the karlotoxin group was structurally characterized, the early literature referred to a family of toxins having a UV maximum near 228 nm (KmTx 1 group) and a second family with a UV maximum near 235 nm (KmTx 2 group).¹⁸ The shift in UV absorbance maximum is a result of chlorination of the terminal olefin.²⁶ The presence of KmTx 1-type toxins has until now been mutually exclusive with the presence of KmTx 2-type toxins, with the former being associated with strains isolated from the Chesapeake Bay and the latter associated with strains isolated from southerly regions of the U.S. East Coast.^{5,6,18,21} Here we report the simultaneous production of KmTx 1-type and KmTx 2-type

Table 1. ¹H NMR and ¹³C NMR Data for Compounds **3–5** in 1:2 Pyridine-*d*₅/MeOH-*d*₄^a

posn	3		4		5	
	δ_C , mult.	δ_H , mult, (J in Hz)	δ_C , mult.	δ_H , mult, (J in Hz)	δ_C , mult.	δ_H , mult, (J in Hz)
1	66.81, CH ₂	3.60, dd (11.1, 4.5); 3.55, m	66.7, CH ₂	3.60, dd (10.9, 4.5); 3.56, m	66.5, CH ₂	3.59, dd (10.9, 4.5); 3.55, m
2	73.0, CH	3.74, m	72.7, CH	3.75, m	72.7, CH	3.75, m
3	37.70, CH ₂	2.31, m; 2.22, m	37.5, CH ₂	2.32, m; 2.23, m	37.5, CH ₂	2.31, m; 2.22, m
4	128.23, CH	5.74, dt (15.5, 7.3)	128.0, CH	5.75, dt (15.4, 7.1)	128.0, CH	5.74, dt (15.1, 7.5)
5	137.11, CH	5.58, dd (15.3, 6.9)	136.9, CH	5.59, dd (15.4, 7.0)	136.8, CH	5.56, dd (15.1, 7.3)
6	73.34, CH	4.05, m	73.1, CH	4.06, m	73.0, CH	4.05, m
7	38.5, CH ₂	1.55, m; 1.49, m	38.3, CH ₂	1.55, m; 1.49, m	38.1, CH ₂	1.55, m; 1.49, m
8	22.82, CH ₂	1.59, m; 1.40, m	22.3, CH ₂	1.59, m; 1.40, m	1.73–1.44, m ^b	1.69, m
9	38.5, CH ₂	1.45, m	38.2, CH ₂	1.45, m	34.9, CH ₂	1.69, m
10	72.02, CH	3.56, m	71.8, CH	3.57, m	79.9, CH	4.49, p (5.6)
11	38.4, CH ₂	1.44, m	38.2, CH ₂	1.44, m	34.9, CH ₂	1.69, m
12	23.17, CH ₂	1.65, m; 1.44, m	22.7, CH ₂	1.65, m; 1.44, m	1.73–1.44, m ^b	1.73–1.44, m
13	39.82, CH ₂	1.48, m	39.6, CH ₂	1.48, m	39.5, CH ₂	1.47, m
14	70.16, CH	3.69, m	70.0, CH	3.69, m	69.9, CH	3.69, m
15	41.04, CH ₂	1.77, m; 1.29, m	40.8, CH ₂	1.77, m; 1.30, m	40.9, CH ₂	1.76, m; 1.28, m
16	33.36, CH	2.09, m	33.2, CH	2.10, m	33.1, CH	2.08, m
17	79.64, CH	3.19, dd (6.1, 3.3)	79.5, CH	3.21, dd (6.0, 4.4)	79.3, CH	3.19, dd (6.1, 3.5)
18	72.82, CH	3.69, m	72.6, CH	3.69, m	72.5, CH	3.70, m
19	31.75, CH ₂	1.71, m; 1.64, m	31.3, CH ₂	1.73, m; 1.65, m	31.5, CH ₂	1.71, m; 1.64, m
20	31.55, CH ₂	1.73, m; 1.44, m	31.1, CH ₂	1.73, m; 1.45, m	31.2, CH ₂	1.71, m; 1.45, m
21	35.03, CH	2.01, m	34.9, CH	2.03, m	34.9, CH	2.02, m
22	73.79, CH	3.85, dd (9.0, 1.75)	73.7, CH	3.87, dd (9.0, 1.75)	73.4, CH	3.87, dd (8.7, 1.7)
23	73.97, CH	3.47, d (9.1)	73.9, CH	3.49, d (9.2)	73.8, CH	3.49, d (9.2)
24	71.53, CH	4.03, m	71.4, CH	4.04, m	71.2, CH	4.03, m
25	35.36, CH ₂	1.77, m; 1.57, m	35.1, CH ₂	1.78, m; 1.59, m	35.0, CH ₂	1.77, m; 1.57, m
26	24.38, CH ₂	1.52, m; 1.46, m	24.2, CH ₂	1.53, m; 1.48, m	24.0, CH ₂	1.53, m; 1.45, m
27	33.07, CH ₂	1.71, m; 1.27, m	32.6, CH ₂	1.73, m; 1.29, m	32.8, CH ₂	1.70, m; 1.28, m
28	36.38, CH	1.78, m	36.1, CH	1.78, m	36.1, CH	1.77, m
29	79.80, CH	3.13, dd (7.1, 2.7)	79.6, CH	3.13, dd (6.7, 2.7)	79.6, CH	3.14, dd (6.8, 2.7)
30	68.36, CH	4.01, m	68.3, CH	4.03, m	68.3, CH	4.03, m
31	34.77, CH ₂	1.92, m; 1.80, m	34.7, CH ₂	1.93, m; 1.81, m	1.94, m; 1.80, m ^b	1.94, m; 1.80, m
32	74.82, CH	4.34, dt (10.7, 2.6)	74.6, CH	4.36, dt (10.4, 2.3)	74.7, CH	4.36, m
33	73.08, CH	3.89, m	72.9, CH	3.91, m	72.8, CH	3.91, m
34	73.1, CH	3.91, dd (7.8, 3.0)	72.9, CH	3.93, dd (7.5, 3.0)	72.9, CH	3.93, dd (7.4, 3.4)
35	71.29, CH	4.10, t (7.9)	71.1, CH	4.13, t (7.8)	71.0, CH	4.11, t (7.8)
36	77.87, CH	3.56, m	77.7, CH	3.58, m	77.6, CH	3.58, m
37	72.75, CH	4.16, m	72.6, CH	4.18, m	72.5, CH	4.18, m
38	31.75, CH ₂	2.16, m; 1.81, m	31.6, CH ₂	2.18, m; 1.83, m	31.9, CH ₂	2.20, m; 1.84, m
39	27.54, CH ₂	2.61, m; 2.25, m	27.4, CH ₂	2.64, m; 2.27, m	27.3, CH ₂	2.62, m; 2.23, m
40	151.7, C		^c		^c	
41	76.82, CH	4.38, d (8.86)	76.6, CH	4.40, d (8.8)	76.5, CH	4.40, d (8.8)
42	74.88, CH	3.52, d (8.7)	74.8, CH	3.52, d (8.8)	74.7, CH	3.53, m
43	70.44, CH	4.22, m	70.8, CH	4.23, m	70.2, CH	4.23, m
44	31.75, CH ₂	2.30, m; 1.62, m	31.6, CH ₂	2.33, m; 1.63, m	31.6, CH ₂	2.32, m; 1.63, m
45	67.18, CH	4.19, m	67.0, CH	4.20, m	66.9, CH	4.21, m
46	68.64, CH	4.31, t (2.0)	68.5, CH	4.34, bs	68.4, CH	4.33, m
47	80.59, CH	3.98, d (10.1)	80.3, CH	4.01, d (10.0)	80.3, CH	4.01, d (10.1)
48	72.02, CH	4.18, m	72.0, CH	4.20, m	72.0, CH	4.20, m
49	74.17, CH	4.59, dd (7.3, 2.9)	74.0, CH	4.62, dd (7.3, 3.1)	74.0, CH	4.60, dd (6.9, 2.7)
50	128.97, CH	5.73, dd (15.5, 9.5)	128.8, CH	5.78, m	128.8, CH	5.76, m
51	135.21, CH	5.81, dt (15.4, 6.3)	135.0, CH	5.82, dt (15.4, 6.0)	134.9, CH	5.80, dt (15.6, 5.9)
52	33.51, CH ₂	1.96, m	33.2, CH ₂	1.96, m	33.2, CH ₂	1.96, m
53	30.1–30.6, CH ₂	1.27, m	30, CH ₂	1.27, m	30, CH ₂	1.27, m
54	30.1–30.6, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.14, m	29–31, CH ₂	1.18–1.14, m
55	30.1–30.6, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
56	30.1–30.6, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
57	30.1–30.6, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
58	30.1–30.6, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
59	30.1–30.6, CH ₂	1.27, m	30, CH ₂	1.27, m	30, CH ₂	1.27, m
60	33.41, CH ₂	1.98, m	33.2, CH ₂	1.98, m	33.2, CH ₂	1.98, m
61	136.25, CH	5.63, dt (15.5, 7.8)	136.9, CH	5.65, dt (15.0, 7.3)	136.0, CH	5.63, dt (14.5, 6.8)
62	132.24, CH	6.00, dd (15.1, 10.6)	127.2, CH	5.97, dd (15.1, 10.9)	131.2, CH	5.99, dd (15.1, 10.7)
63	138.57, CH	6.27, dt (17.0, 10.2)	135.0, CH	6.39, dd (12.9, 11.0)	138.4, CH	6.28, dt (16.8, 10.1)
64	115.2, CH ₂	5.03, d (15.0); 4.88, d (10.4)	119.0, CH	6.19, d (13.0)	114.8, CH ₂	5.03, d (16.4); 4.88, d (10.5)
65	17.72, CH ₃	0.98, d (6.5)	17.6, CH ₃	0.99, d (6.3)	17.5, CH ₃	0.97, d (6.1)
66	13.56, CH ₃	0.97, d (6.5)	13.3, CH ₃	0.98, d (6.5)	13.3, CH ₃	0.98, d (6.2)
67	16.86, CH ₃	0.92, d (6.7)	16.6, CH ₃	0.92, d (6.7)	16.6, CH ₃	0.91, d (6.4)
68	113.03, CH ₂	5.11, s; 5.01, s	112.7, CH ₂	5.11, s; 5.01, s	112.7, CH ₂	5.12, s; 5.02, s

^a Chemical shifts referenced to residual methanol-*d*₄ signals at 3.30 and 49.0 ppm for ¹H and ¹³C, respectively. ^b Cross-peak in HSQC not visible due to low intensity. ^c Not determined due to low sample availability.

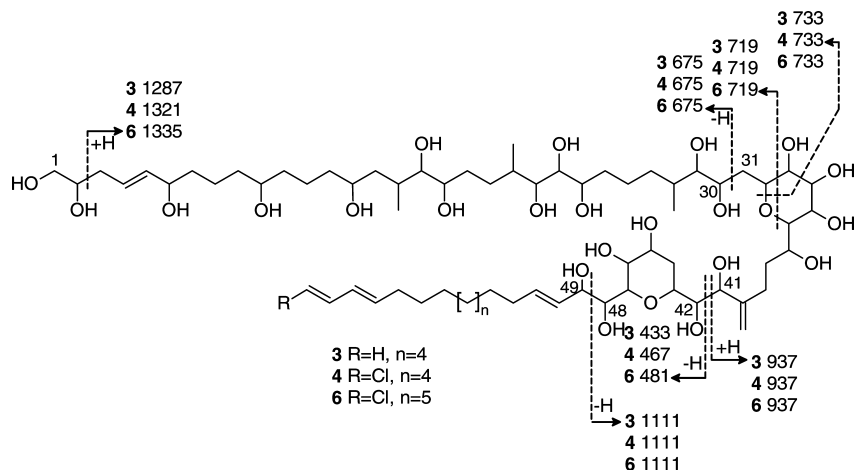


Figure 1. Fragmentation pathways for **3**, **4**, and **6**. For each compound $[M + Na]^+$ was selected as precursor, and all fragment masses include sodium ions. Note that the fragments resulting from scission of C-41/C-42 and from scission of C-48/C-49 indicate that differences between the structures occur in the lipophilic arm.

toxins by a single strain. This suggests that the ability to produce one or the other type of toxin is not as genetically distinct as previously thought and that, under the right conditions, a single strain can produce both. Hence, rather than refer to the chlorinated analogues of KmTx 1 and KmTx 3 (**3**) as KmTx 2 derivatives, we prefer to name them as chlorinated derivatives of their parent compounds, holding to the tradition of assigning unique parent compound names to unique carbon skeletons within a family. The compound we have named KmTx 3 (**3**) has a similar molecular weight and MS-MS fragmentation pattern to hydroxy KmTx 1-1.²¹ Re-examination of previous UV and MS spectra and comparison to those reported here identifies the minor component originally named KmTx 1-2⁵ as 64-*E*-chloro-KmTx 3 (**4**), with 65-*E*-chloro-KmTx 1 (**6**) being evident in the chromatograms for other Chesapeake Bay strains. Although the existence of sulfonated karlotoxins has been discussed before,^{5,21} this report represents the first characterization of the site of sulfonation.

The trends for hemolytic activity of **3–7** appear to be consistent with past reports for both karlotoxins and amphidinols. As we noted previously,⁴ amphidinols exhibit an increase in potency with longer lipophilic arms.^{27–29} The same trend appears in karlotoxins, with KmTx 2 (**2**) being nearly an order of magnitude less potent than KmTx 1 (**1**), which has a lipophilic arm that is two carbons longer.^{4,5} Chlorination of the terminal diene had little (**4** vs **3**) or no (**6** vs **1**) enhancing effects on potency, suggesting that the difference in potency between KmTx 1 (**1**) and KmTx 2 (**2**) can be entirely attributed to lipophilic arm length. The higher potency of KmTx 1 with respect to KmTx 2 is also reflected in the comparable efficacies of each in immobilizing organisms that *K. veneficum* preys upon, suggesting similarities in mechanism between hemolysis and prey immobilization.¹⁰ By contrast, KmTx 1 and KmTx 2 are comparably effective at damaging zebrafish gills.⁹ As previously observed with amphidinols,^{23,24,30} sulfonation of the polyol arm in the karlotoxins leads to dramatic reductions in potency. Thus sulfonation of karlotoxins could represent a strategy for safe storage, perhaps supplementing the resistance to karlotoxin toxicity provided by the sterols of *K. veneficum*.^{13–15}

Another unusual aspect of karlotoxins occurs in the length of the lipophilic arm (C-50–C-64 in **3**). In all other amphidinol-like compounds, there is an even number of carbons in this arm. From the two biosynthetic labeling studies that have been reported for the amphidinols, this part of the biosynthetic pathway appears highly conserved.^{25,31} Extrapolating from these labeling results, karlotoxins with an even number of carbons in the lipophilic side chain (18-carbon KmTx 1 group; 16-carbon KmTx 2 group) are most likely assembled from intact acetate units with the inclusion of two deleted

units. However, the KmTx 3 group contains a side chain with an odd number of carbons, suggesting the biosynthetic pathway leading to this group differs by the inclusion of an additional carbon deletion step compared with the KmTx 1 group.³² Also unique to the karlotoxins is the chlorination of the terminal diene. Halogenation of dinoflagellate natural products is rare, though a terminal chloroalkene is present in the prymnesins produced by the phytoflagellate *Prymnesium parvum*.^{33,34} Each of these distinctive aspects of chemical structure and biological activity serves to distinguish the karlotoxins as a fascinating family of compounds.

Experimental Section

General Experimental Procedures. Optical rotations were acquired on a Randolph Research Analytical Autopol III automatic polarimeter. UV spectra were acquired on a Beckman DU 640B spectrophotometer. IR data were measured using a Madison Cygnus 100 spectrometer. All NMR spectra were acquired in 1:2 pyridine-*d*₅/MeOH-*d*₄ on a 500 MHz Bruker Avance spectrometer with a 1.7 mm TXI probe. NMR data were analyzed using Topspin 2.0 (Bruker Biospin, Inc.). LC/MS data for monitoring the fractionation process were acquired on a Waters/Micromass ZQ detector with an ESI interface coupled to an Agilent 1100 HPLC system with a Waters XTerra MS C₈ column (2.1 × 30 mm) and a gradient mobile phase system consisting of CH₃CN and H₂O, each containing 0.1% acetic acid. High-resolution mass spectra were obtained using a Waters QToF Ultima mass spectrometer with an ESI interface by the Mass Spectrometry Laboratory at the University of Illinois at Urbana–Champaign. Enhanced product ion MS/MS experiments were conducted on an Applied Biosystems QTrap linear ion trap quadrupole LC/MS/MS system. Collision-induced dissociation was accomplished using an ion source potential of 4500 V, a collision energy of 130 eV, and a high gas pressure in the collision cell. Preparative HPLC was accomplished using a system with two Waters 515 HPLC pumps, a gradient controller, and a Waters 2487 dual-wavelength UV detector.

Hemolytic Assay. The hemolytic assay was performed using human erythrocytes obtained from the American Red Cross. Erythrocytes were washed, centrifuged, and suspended to a concentration of $(4.2–4.7) \times 10^4$ cells/mL using ELA buffer.³⁵ Equal volumes (125 μ L) of erythrocyte suspension and test solution (compound or control in ELA buffer) were combined in 96-well V-bottom microtiter plates (Costar). Each sample was tested in triplicate. Sealed plates were incubated at 4 °C for 24 h and centrifuged, and lysis of erythrocytes was measured by optical density at 415 nm for the supernatant. For each concentration tested, the activity was measured as a percentage compared to a positive control using saponin from quillaja bark (20–35% saponin content; Sigma, Inc.). For each series of concentrations, EC₅₀ values were estimated using nonlinear regression analysis in GraphPad Prism.

Biological Material. The dinoflagellate *Karlodinium veneficum* (CCMP 2936) was originally isolated from the Delaware Inland Bays, U.S. The particular sample used was a gift from Kathy Coyne

Table 2. ¹H NMR and ¹³C NMR Data for Compounds **6** and **7** in 1:2 Pyridine-*d*₅/MeOH-*d*₄^a

posn	6		7	
	δ _C , mult.	δ _H , mult. (<i>J</i> in Hz)	δ _C , mult.	δ _H , mult. (<i>J</i> in Hz)
1	66.6, CH ₂	3.60, dd (10.9, 4.5); 3.56, m	66.6, CH ₂	3.62, dd (10.9, 4.1); 3.55, m
2	72.8, CH	3.75, m	72.7, CH	3.76, m
3	37.5, CH ₂	2.31, m; 2.23, m	37.5, CH ₂	2.30, m; 2.22, m
4	127.9, CH	5.75, dt (15.6, 7.1)	127.8, CH	5.74, dt (15.5, 7.0)
5	136.9, CH	5.59, dd (15.6, 7.0)	136.8, CH	5.57, dd (15.5, 7.1)
6	73.1, CH	4.06, m	72.9, CH	4.05, m
7	38.2, CH ₂	1.57, m; 1.51, m	38.1, CH ₂	1.54, m
8	^b	1.59, m; 1.40, m	21.8, CH ₂	1.58, m; 1.53, m
9	38.2, CH ₂	1.44, m	35.0, CH ₂	1.69, m
10	71.7, CH	3.57, m	79.9, CH	4.49, m
11	38.2, CH ₂	1.44, m	35.0, CH ₂	1.69, m
12	22.7, CH ₂	1.65, m; 1.44, m	21.9, CH ₂	1.62, m; 1.49, m
13	39.6, CH ₂	1.48, m	39.4, CH ₂	1.47, m
14	69.9, CH	3.70, m	69.8, CH	3.70, m
15	40.9, CH ₂	1.77, m; 1.29, m	40.8, CH ₂	1.77, m; 1.27, m
16	33.2, CH	2.10, m	33.1, CH	2.09, m
17	79.5, CH	3.20, dd (5.8, 3.6)	79.3, CH	3.19, dd (6.1, 3.2)
18	72.5, CH	3.70, m	72.4, CH	3.70, m
19	31.3, CH ₂	1.73, m; 1.64, m	31.2, CH ₂	1.72, m; 1.63, m
20	31.4, CH ₂	1.73, m; 1.45, m	31.3, CH ₂	1.72, m; 1.45, m
21	34.8, CH	2.03, m	34.8, CH	2.02, m
22	73.6, CH	3.87, dd (9.2, 1.5)	73.7, CH	3.86, dd (8.6, 1.5)
23	73.8, CH	3.49, d (8.9)	73.7, CH	3.50, d (8.8)
24	71.4, CH	4.04, m	71.4, CH	4.05, m
25	35.1, CH ₂	1.79, m; 1.58, m	35.1, CH ₂	1.77, m; 1.58, m
26	24.3, CH ₂	1.54, m; 1.47, m	24.1, CH ₂	1.53, m; 1.46, m
27	32.7, CH ₂	1.73, m; 1.29, m	32.7, CH ₂	1.72, m; 1.28, m
28	36.2, CH	1.79, m	36.1, CH	1.79, m
29	79.7, CH	3.14, dd (7.3, 2.9)	79.6, CH	3.15, dd (7.0, 2.9)
30	68.2, CH	4.03, m	68.1, CH	4.03, m
31	34.7, CH ₂	1.94, m; 1.81, m	34.6, CH ₂	1.94, m; 1.80, m
32	74.6, CH	4.36, dt (10.7, 2.7)	74.6, CH	4.36, m
33	72.8, CH	3.91, m	72.8, CH	3.91, m
34	73.0, CH	3.94, dd (7.8, 2.7)	72.9, CH	3.93, dd (8.0, 2.7)
35	71.1, CH	4.13, t (8.0)	71.1, CH	4.12, m
36	77.7, CH	3.57, m	77.5, CH	3.58, m
37	72.6, CH	4.18, m	72.5, CH	4.18, m
38	31.8, CH ₂	2.18, m; 1.84, m	31.6, CH ₂	2.16, m; 1.83, m
39	27.3, CH ₂	2.64, m; 2.28, m	27.3, CH ₂	2.63, m; 2.25, m
40	^c		^c	
41	76.6, CH	4.41, d (8.9)	76.5, CH	4.40, d (8.9)
42	74.9, CH	3.53, d (9.1)	74.7, CH	3.54, m
43	70.2, CH	4.23, m	70.2, CH	4.23, m
44	31.6, CH ₂	2.33, m; 1.64, m	31.4, CH ₂	2.31, m; 1.64, m
45	67.0, CH	4.19, m	66.9, CH	4.21, m
46	68.4, CH	4.34, bs	68.4, CH	4.35, bs
47	80.4, CH	4.01, d (10.0)	80.3, CH	4.01, d (10.1)
48	72.1, CH	4.20, m	71.9, CH	4.20, m
49	73.9, CH	4.62, dd (7.1, 2.7)	73.9, CH	4.61, dd (7.4, 2.5)
50	128.9, CH	5.76, m	128.9, CH	5.74, m
51	134.9, CH	5.82, dt (15.4, 6.3)	134.9, CH	5.82, dt (15.1, 6.0)
52	33.2, CH ₂	1.96, m	33.2, CH ₂	1.96, m
53	30, CH ₂	1.26, m	30, CH ₂	1.26, m
54	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
55	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
56	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
57	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
58	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
59	29–31, CH ₂	1.14–1.18, m	29–31, CH ₂	1.14–1.18, m
60	30, CH ₂	1.27, m	30, CH ₂	1.26, m
61	33.2, CH ₂	1.98, m	33.2, CH ₂	1.96, m
62	136.9, CH	5.65, dt (14.6, 7.0)	136.0, CH	5.63, dt (14.8, 7.0)
63	127.2, CH	5.97, dd (15.1, 11.3)	131.2, CH	6.00, dd (15.3, 11.3)
64	134.9, CH	6.39, dd (12.8, 11.0)	138.3, CH	6.27, dt (17.4, 10.7)
65	119.0, CH	6.19, d (13.1)	114.8, CH ₂	5.03, d (16.8); 4.88, d (10.1)
66	17.5, CH ₃	0.98, d (6.4)	17.5, CH ₃	0.96, d (6.2)
67	13.3, CH ₃	0.98, d (6.4)	13.3, CH ₃	0.98, d (6.5)
68	16.7, CH ₃	0.92, d (6.4)	16.6, CH ₃	0.91, d (6.5)
69	112.7, CH ₂	5.11, s; 5.01, s	112.7, CH ₂	5.12, s; 5.01, s

^a Chemical shifts referenced to residual methanol-*d*₄ signals at 3.30 and 49.0 ppm for ¹H and ¹³C, respectively. ^b Cross-peak in HSQC not visible due to low intensity. ^c Not determined due to low sample availability.

(University of Delaware, Lewes, DE). Cultures were grown in 10 L batches (40 L total) using seawater from Indian River, DE, after

sterilization (autoclaving at 121 °C, 15 psi) and dilution to a salinity of 15 psi using doubly deionized water. The culture media were

Table 3. Hemolytic Activity of **1** and **3–7**

compound	EC ₅₀ (nM)
1	63 ^a
3	200
4	110
5	2400
6	56
7	300

^a Taken from ref 4.

supplemented with f/2-Si nutrients.³⁶ Cultures were grown using a 14/10 h light/dark cycle at 20 °C and were harvested using GF/F filters (Whatman, Inc.).¹ The filtrate was subject to solid-phase extraction using BakerBond C₁₈ (J.T. Baker, Inc.) and the hydrophobic material recovered using MeOH and 1:1 acetone/MeOH (v/v).

Isolation. The filtrate extract (100 mg) was fractionated by flash chromatography (1.2 × 19 cm) using BakerBond C₁₈ (40 μm, J.T. Baker, Inc.) and a step gradient consisting of 20 mL each of 30%, 45%, 60%, and 80% aqueous MeOH and 50 mL of MeOH. Under these conditions, sulfonated karlotoxins eluted at 60–80% MeOH, whereas the other karlotoxins eluted at 80–100% MeOH. The latter fraction was further fractionated using a Waters Sunfire C₁₈ column (4.6 × 150 mm; 3.5 μm) and isocratic elution with 62% water, 38% MeCN at 0.8 mL/min to yield **3** (0.7 mg), **4** (0.4 mg), and **6** (0.2 mg). The sample containing sulfonated karlotoxins was fractionated using a Develosil TMS-UG-5 C₁ column (4.6 × 250 mm) using isocratic elution with 64% 2 mM ammonium acetate and 36% MeCN at 0.8 mL/min to yield **5** (0.3 mg) and **7** (0.5 mg).

KmTx 3 (3): colorless oil (0.7 mg); [α]_D²⁵ +14 (c 1.4, MeOH); UV (MeOH) λ_{max} (log ε) 228 (4.2), 253 (3.7), 259 (3.7) nm; IR (film) ν_{max} 3365, 2920, 2851, 1073 cm⁻¹; ¹H and ¹³C NMR Table 1; HRESIMS (+) m/z 1325.8534 (calcd for C₆₈H₁₂₅O₂₄, 1325.8561; Δ = -2.0 ppm).

64-E-Chloro-KmTx 3 (4): colorless oil (0.4 mg); [α]_D²⁵ +2 (c 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 233 (4.1), 238 (4.1) nm; ¹H and ¹³C NMR Table 1; HRESIMS (+) m/z 1359.8165 (calcd for C₆₈H₁₂₄ClO₂₄, 1359.8171; Δ = -0.4 ppm).

10-O-sulfo-KmTx 3 (5): colorless oil (0.3 mg); [α]_D²⁵ +3 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 228 (4.1), 258 (3.8) nm; ¹H and ¹³C NMR Table 1; HRESIMS (+) m/z 1405.8108 (calcd for C₆₈H₁₂₅O₂₇S, 1405.8129; Δ = -1.5 ppm).

65-E-Chloro-KmTx 1 (6): colorless oil (0.2 mg); [α]_D²⁵ +9 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 233 (4.2), 238 (4.2); ¹H and ¹³C NMR Table 2; HRESIMS (+) m/z 1373.8344 (calcd for C₆₉H₁₂₆ClO₂₄, 1373.8328; Δ = +1.2 ppm).

10-O-sulfo-KmTx 1 (7): colorless oil (0.5 mg); [α]_D²⁵ +3 (c 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 229 (4.2), 253 (4.0), 258 (4.0) nm; ¹H and ¹³C NMR Table 2; HRESIMS (+) m/z 1419.8248 (calcd for C₆₉H₁₂₇O₂₇S, 1419.8285; Δ = -2.6 ppm).

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Supporting Information Available: 1D and 2D NMR spectra for compounds **3–7**; EPI spectra for compounds **3**, **4**, and **6**; high-resolution mass spectra for compounds **3–7**; hemolytic assay EC₅₀ curves for compounds **3–7**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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