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Karmitoxin: An Amine-Containing Polyhydroxy-Polyene Toxin from the Marine Dinoflagellate Karlodinium armiger

Silas Anselm Rasmussen,† Sofie Bjørnholt Binzer,‡ Casper Hoeck,§ Sebastian Meier,§ Livia Soman de Medeiros,† Nikolaj Gedsted Andersen,‖ Allen Place,∇ Kristian Fog Nielsen,† Per Juel Hansen,§*
Thomas Ostenfeld Larsen†*

†Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads building 221, DK-2800 Kgs. Lyngby, Denmark

‡Marine Biological Section, Department of Biology, University of Copenhagen, Strandpromenaden 5, DK-3000 Helsingør, Denmark

§Department of Chemistry, Technical University of Denmark, Kemitorvet building 207, DK-2800 Kgs. Lyngby, Denmark

‖Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 05508-000, Brazil

‖National Veterinary Institute, Technical University of Denmark, Bülowsvæj 27, DK-1870 Frederiksberg C, Denmark

∇University of Maryland Center for Environmental Research, Institute of Marine and Environmental Technology, 701 E. Pratt St., Baltimore, MD 21202, USA
Abstract

Marine algae from the genus *Karlodinium* are known to be involved in fish killing events worldwide. Here we report for the first time the chemistry and bioactivity of a natural product from the newly described mixotrophic dinoflagellate *Karlodinium armiger*. Our work describes the isolation and structural characterization of a new polyhydroxy-polyene named karmitoxin. The structure elucidation work was facilitated by use of $^{13}$C enrichment and high-field 2D NMR spectroscopy, where $^1$H-$^{13}$C long-range correlations turned out to be very informative. Karmitoxin is structurally related to amphidinols and karlotoxins, however it differs by containing the longest carbon-carbon backbone discovered for this class of compounds, as well as a primary amino group. Karmitoxin showed potent nanomolar cytotoxic activity in an RTgill-W1 cell assay as well as rapid immobilization and eventual mortality of the copepod *Acartia tonsa*, a natural grazer of *K. armiger*. 
Marine algae from the cosmopolite dinoflagellate genus *Karlodinium* have been implicated in several fish killing events. Recently, the responsible fish-killing toxin from *K. veneficum*, named karlotoxin 1 (1), was isolated and its structure characterised by van Wagoner *et al.* Subsequently, the absolute configuration of karlotoxin 2 (2) was assigned in a study by Peng *et al.* Several other congeners have since been reported. Common for karlotoxins are that they are large linear amphipathic compounds consisting of a carbon backbone containing between 60-64 carbon atoms, with two hydroxylated tetrahydropyran rings, one of which has an attached lipophilic arm, while the other has a polyhydroxy arm. These toxins are related to amphidinols (e.g. amphidinol 3 (4)), originally isolated from the dinoflagellate *Amphidinium klebsii*, compounds that have been shown to exhibit both cytotoxic and antifungal activity. In addition the amphidinol analogue, carteraol E, has been shown to be ichthyotoxic, but the experimental conditions were not stated.

Karlotoxins have also been indicated to be produced by *K. conicum*, a recently discovered species from the Australian Ocean. Thus, Mooney *et al.* screened several Australian strains of *K. veneficum* and *K. conicum* for haemolytic activity and for their capabilities to produce karlotoxin-like compounds. Based on UV and mass spectrometric data it was found that strains of *K. veneficum* produced the well-known karlotoxin 2 as well as two unknown karlotoxin-2 related compounds, whereas *K. conicum* was reported to produce two karlotoxin-like compounds (KmTx 9/KcTx 1) with low haemolytic activity. In addition, karlotoxins have also been reported in the East China Sea.

The mode-of-action of karlotoxins has been shown to be related to the disruption of the cell membrane by specific binding to cholesterol, thus creating pores in the membrane similar to that of the amphidinols. This behaviour has been attributed to the hairpin conformation of this compound class when binding to cholesterol in bilayer membranes. *K. veneficum*’s principle membrane sterol is gymnodinosterol which renders it immune to its own toxin. In addition, karlotoxin is also one of the few toxins that have been shown to pose allelochemical properties to other phytoplankton and thus also functions as a grazing deterrent.

Recently, a new *Karlodinium* species, *K. armiger* was isolated in the Mediterranean Sea. *K. armiger* has been associated with several reported fish kills and is therefore considered to be an ichthyotoxic species. Like, *K. veneficum*, *K. armiger* is mixotrophic, because it relies on both prey feeding and photosynthesis. However, *K. armiger* grows very slowly without prey (0.01-0.06
divisions per day)\textsuperscript{17} on a nitrate based nutrient medium such as f/2, unlike \emph{K. veneficum} which grows well in such nutrient media (0.3 division per day), although better in the presence of prey.\textsuperscript{18}

Both \emph{K. armiger} and \emph{K. veneficum} are peduncle feeders (tube feeders). Upon identification of a potential prey they apply a capture filament (a harpoon) and subsequently immobilize the prey, allowing them to suck out prey nutrients or pull the prey into a food vacuole for digestion. Both species can immobilize prey of a wide range of different sizes, such as algae, copepods and other metazoans.\textsuperscript{19–22} For instance, it has been shown that cultures of \emph{K. armiger} (15 μm) are able to immobilize the much larger copepod, \emph{Acartia tonsa} (1 mm), within minutes.\textsuperscript{23} Karlotoxins from \emph{K. veneficum} have been shown to be involved in prey immobilization,\textsuperscript{14,19} however, apparently not as effective towards copepods, such as \emph{A. tonsa}, that respond by a decreased food uptake.\textsuperscript{20,21} In line with this another study reported that whole culture and filtrates of \emph{K. corsicum} cause paralysis of the female copepod \emph{A. granii}.\textsuperscript{24}

In this paper we have identified and characterized a new karlotoxin-like compound, that we have named karmitoxin (3). Karmitoxin is longer than any previously described karlotoxin-like compounds. Furthermore, karmitoxin contains an amino group, a feature not previously seen for such compounds. The toxicity of karmitoxin proved to be in the nanomolar range, both when tested in a RTgill-W1 cell assay,\textsuperscript{25} and towards the copepod, \emph{A. tonsa}. 
The initial investigation of a *K. armiger* extract identified the presence of a compound with a protonated molecule ([M+H]<sup>+</sup> of <em>m/z</em> 1386.8875) which combined with the isotopic pattern indicated an elemental composition of C<sub>73</sub>H<sub>127</sub>NO<sub>23</sub>. The compound had a maximum UV absorption at 226 nm similar to that of karlotoxin 1 (1). In order to isolate this compound in quantities allowing for NMR characterization, two large-scale cultivations (60 L natural abundance and 60 L enriched with NaH<sup>13</sup>CO<sub>3</sub>) of *K. armiger* were performed.

Although, the *K. armiger* strain K-0668 usually grows very slowly without the addition of prey, such as the cryptophyte *Rhodomonas salina*, it was found that the growth rate could be increased to ~0.3 division per day, simply by the addition of ammonium, although, the addition of
NH₄⁺ should be carefully controlled, due to its equilibrium with the toxic NH₃ at higher pH. Hence, first we had to establish the concentration of ammonium that could be spiked to the cultures and that would generate the wanted cell concentration without being toxic. We found that spiking of the *K. armiger* culture with 50 µM ammonium was sufficient in order to grow *K. armiger* up to a cell density of ~15×10⁵ cells·mL⁻¹. By utilizing the ammonium spiking, two large-scale cultivations were carried out, one being in normal natural ¹³C abundance medium, whereas the other cultivation was performed in the presence of 150 mg L⁻¹ NaH¹³CO₃. Following cultivation, the compound, hereafter named karmitoxin (3), was isolated from the supernatants using standard semi-preparative reversed-phase chromatography.

The structure of karmitoxin was elucidated by extensive analysis of homo- and heteronuclear high-field NMR data. The HSQC spectrum showed two methyl groups, 21 aliphatic methylene groups, 25 likely oxygenated carbon atoms, 15 olefinic protonated carbons two of which have geminal protons. In addition, the HMBC spectrum and ¹³C spectrum revealed one quaternary olefinic carbon as well as a carbonyl carbon atom, altogether suggesting a total of nine double bonds. With a calculated number of double bond equivalents of 11 this left us with two rings to account for.

![Figure 1. Structure of the elucidated backbone of karmitoxin, where bold red bonds illustrate the parts that were assembled by long-range ²JCH correlation and blue bold bonds show the parts assembled by COSY/TOCSY correlations, together with key HMBC correlations connecting the different parts.](image)

The structure elucidation was mainly carried out on the 30 % uniformly ¹³C-enriched sample by analysis of HSQC-TOCSY, HSQC and HMBC data. COSY and NOESY data, were acquired on the natural abundance sample. The elucidation of karmitoxin was eased by the observation that most HSQC correlations in the ¹³C-enriched material exhibited ²JCH long-range correlations, allowing sequential spectral assignment from one carbon atom to the adjacent carbon atoms.²⁶ This approach established the majority of the carbon backbone: C-4 to C-13, C-15 to C-26, C-29 to C-36, C-38 to C-42, C-43 to C-44, C-46 to C-50 and C-51 to C-70 (Figure 1). The connections that
could not be established by these long-range $^{2}J_{CH}$ couplings were subsequently established by COSY, HSQC-TOCSY and HMBC correlations.. The connection H-1 to H-3 was identified with COSY data and was further verified by HMBC correlations seen from H-1 to C-2 and C-3. No TOCSY correlations were observed for H-26 to H-29 and this linkage was hence established by HMBC correlation observed from H-71 to C-26, C-27 and C-28 and HMBC correlations observed from H-28 to C-27 and C-29. The sequence was confirmed by very weak COSY correlations. The connection from C-36 to C-38 was identified by TOCSY and confirmed by HMBC correlations observed from H-37 to C-35, C-36, and C-37. The connection C-42 to C-43 was established by TOCSY and an HMBC correlation from H-44 to C-42. The quaternary carbon C-45 was identified by a long-range $^{2}J_{CH}$ coupling from the geminal protons H-73 to C-45. The positioning of C-45 in between C-44 and C-46 was confirmed by HMBC correlations from H-73 to C-44 and C-46. The connection C-50 to C-51 was established by TOCSY and confirmed by HMBC correlations from H-49 to C-50 and C-51. The two oxygenated carbons, C-38 and C-39 were observed to exhibit near-identical proton and carbon chemical shifts ($\delta_{H}$ 3.72, $\delta_{C}$ 71.6). This was confirmed by a small chemical shift difference (1 Hz at 800 MHz field, i.e. 1 ppb) observed between the COSY cross peaks of H-37/H-38 and H-39/H-40 (Figure S9). All 1,2-disubstituted carbon-carbon double bonds were found to be of the $E$-configuration, based on large coupling constants ($^{3}J_{HH} > 15$ Hz).

As deduced by the number of double bond equivalents and the excess number of presumed oxygen atoms as deduced from the HSQC data, we suggest karmitoxin to incorporate two cyclic ethers as observed for the karlotoxins. The ring was formed by the ether linkage between C-37 and C-41, established by a HMBC correlation observed from H-41 to C-37 (Figure S8). Similarly a second cyclic ether linkage between C-48 and C-52, could be established by an HMBC correlation from H-52 to C-48 (Figure S8). As indicated by the HRMS analysis, karmitoxin contained a nitrogen atom. It was indicated to be a primary or secondary amine due to its positive reaction with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ Tag). The positioning of the amino group was based on the chemical shift observed for H-1 ($\delta_{H}$ 3.54) and the shielded C-1 shift ($\delta_{C}$ 40.8), both very characteristic of a primary amine.

Elucidation of the exact length of the aliphatic chain (C-55 to C-70) was based on the unassigned C$_{4}$H$_{8}$ atoms that remained after all other signals had been accounted for in the HSQC and HMBC spectra. In the TOCSY and COSY spectra, H-56 and H-66 showed correlations to a strong proton chemical shift value ($\delta_{H}$ 1.32) that displayed a large integral in the $^{1}$H NMR spectrum.
In addition, the $^{13}$C spectrum also revealed several signals around 29 ppm. Hence, we infer that the missing C-H atoms are a chain of four successive CH$_2$ units generating a large aliphatic chain similar to what has been reported for the structurally related karlotoxins.$^2$

Table 1. NMR Spectroscopic Data ($^1$H 800 MHz, $^{13}$C 200 MHz CD$_3$OD) for Karmitoxin (3)$^a$

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<th>$\delta$C</th>
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The relative configurations of the two tetrahydropyran rings were determined based on the observed coupling constants and qualitative NOESY correlations, which showed that both the tetrahydropyran rings C-37 to C-41 and C-48 to C-52 had adopted chair conformations (Figure 2). The tetrahydropyran ring C-37 to C-41 showed large coupling constants ($^3J_{H-40,H-41} = 12$ Hz and $^3J_{H-39,H-40} = 12$ Hz) indicating the anti-orientation for these two proton pairs and an axial orientation of these protons. Furthermore, an NOE was observed for H-41/H-39 confirming the axial orientation of the two protons. A small coupling constant (5 Hz) was observed for $^3J_{H-37,H-38}$, suggesting the gauche conformation, and thus the equatorial orientation of H-38 and H-37. In the second
tetrahydropyran ring (C-48 to C-52), large coupling constants were observed ($^3J_{H-48,H-49a} = 12$ Hz and $^3J_{H-49a,H-50} = 12$ Hz), suggesting that these protons had adopted an axial orientation. The observed NOE between H-48 and H-50 suggested the axial orientation of these protons. Finally, a small coupling constant (3.5 Hz) was observed for $^3J_{H-51,H-52}$, suggesting the equatorial orientation of both protons.

Comparison of the $^{13}$C and $^1$H chemical shifts of the conserved parts of karmitoxin (C-26 to C-54) to those reported for karlotoxin 2 shows almost identical values, expect for C-26. The chemical shift of this methine group, instead resembles that seen in amphidinol 3, in good agreement with the presence of a β positioned hydroxy group (at C-24 in karmitoxin) in both compounds, which is not present in any of the karlotoxins. Altogether, this indicates that the relative configurations of the stereocenters in the conserved part of karmitoxin are similar to the reported configurations of the karlotoxins.12

Recently, the DP4 probability analysis was performed on the polyol arm of new karlotoxin analogues, in order to obtain stereochemical information from experimental and theoretical $^1$H and $^{13}$C chemical shifts.12,27 This method was attempted utilized on karmitoxin, but yielded conflicting answers to the relative configuration of the flexible part (C-1 to C-25), which is thus not considered solved. For data and a full discussion on the difficulties and appropriateness in applying the DP4 probability analysis to such very flexible system, see the Supporting Information.

The biosynthesis of the karmitoxin-related amphidinols has been shown to be catalysed by polyketide synthases by stable isotope incorporation studies using $^{13}$C labelled acetate.28,29 It has been suggested that the starter unit is likely glycolate, as the terminal C-1 and C-2 are not enriched upon $^{13}$C-acetate cultivation.30 Most of the structural differences between the amphidinols and karlotoxins are observed in the polyol side “arm”, where the differences in the length of the “arm” is likely to be explained by different programming of PKS modules of the PKS.28 Here, we speculate that the starter unit of the biosynthesis of karmitoxin is the amino acid glycine. The use of glycine in the assembly of nitrogen containing polyketides, has been shown in the case of the imine containing 13-desmethyl spirolide C, isolated from the dinoflagellate Alexandrium ostenfeldii.31 Three stable isotope incorporation experiments were conducted to test this hypothesis. We supplemented K. armiger with either 1 mM 1-$^{13}$C-glycine, or 1 mM $^{15}$N-glycine for 10 days and 0.5 mM 1,2-$^{13}$C-glycine for 10 days. Although, we observed a clear increase of the A+1 isotopomer, we could not detect any increase of the A+2 in case of the 1,2-$^{13}$C-glycine, which would be
expected if glycine was indeed incorporated. Thus this simple experiment did not lead to further insights into the biosynthetic origin of the amine group in karmitoxin.

The purified toxin was tested both using a rainbow trout gill cell assay (RTgill-W1) as well its ability to immobilize the copepod, *A. tonsa*. Using a calibrated karmitoxin solution it was found to lyse RTgill-W1 cells in a dose-response manner with an LC$_{50}$ value of 125±1 nM (Figure 4A) By utilizing fluorescence tagging, similar to what recently has been reported for the derivatization of B-type of prymnesins, we observed an similar LC$_{50}$ value in the RTgill-W1 assay (99±11 nM) (Figures S10).$^{32}$

When evaluating the purified karmitoxin towards female adult copepods, *A. tonsa*, a rapid immobilization was observed after 2 h, where the copepods did not move or make any “escape jumps”, at the highest tested concentration (1225-2450 nM). Most of the copepods were dead after exposure to 2450 nM of karmitoxin for 4 h, while all copepods were immobilized at a concentration of 1225 Nm of karmitoxin. The 24 h LC$_{50}$ was calculated to 400±100 nM (Figure 4B). This is in good agreement with the identified concentration of karmitoxin in culture (288–432 nM).

![Figure 3](image)

*Figure 3.* Dose-response curve for karmitoxin tested in the RTgill-W1 cell assay (top) after 3 h of exposure in seven replicates. The LC$_{50}$ (3 h) value was calculated to 125±1 nM. Dose-response of the mortality of *A. tonsa* when exposed to karmitoxin (24 h) (bottom) in five replicates. LC$_{50}$ of karmitoxin on *A. tonsa* was calculated to 400±100 nM.

Under physiological conditions the amine of karmitoxin is protonated and we speculate that this could lead to an increased uptake into the cells and perhaps an increased interaction of karmitoxin with negatively charged phospholipids on cell membranes, given the proposed hairpin
In addition, karmitoxin contains a ketone carbonyl also present in karlotoxin-8 and -9 and cartera E, lingshuiol and karatungiols A and B isolated from *Amphidinium* sp. Furthermore, it is also noted that karmitoxin has the longest unbranched carbon-carbon chain discovered in this class of compounds, such as the amphidinols and karlotoxins. The length of the lipophilic arm of this class of compounds is important for haemolytic activity and karmitoxin also exhibits the same length as KmTx1 (C₁₈), which alongside KmTx8 (C₁₆) are the karlotoxins with the highest reported haemolytic activity of rainbow trout erythrocytes. From a toxicity perspective we have shown that karmitoxin is cytotoxic in the mid-nanomolar range in the RTgill-W1 cell assay (Figure 3) and given the structural similarity to karlotoxins, we hypothesize that this toxin also will exhibit ichthyotoxic activity.

In conclusion, we have described the structure of a new structurally unique karlotoxin-like compound named karmitoxin. Future degradation experiments will determine whether the absolute configuration of karmitoxin shares the same absolute configuration of either karlotoxin-2 (2) or the antipodal configuration described for amphidinol-3 (4).

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** The optical rotation was measured in MeOH using a PerkinElmer Model 241 Polarimeter. The UV spectra was measured on a Dionex Ultima 3000 UHPLC. NMR spectra were recorded in CD₃OD (99.96 atom %, Sigma-Aldrich). All data were acquired on a Bruker Avance II 800 MHz spectrometer equipped with a TCI Z-gradient CryoProbe and an 18.7 T magnet (Oxford Magnet Technology). Standard 1D proton, 2D NOESY, 2D COSY and 2D TOCSY spectra were recorded on the natural abundance sample, whereas 1D carbon and heteronuclear 2D experiments were recorded on the ¹³C-enriched version at 293 K. These experiments encompass HSQC, HMBC, HSQC-TOCSY, HSQC-NOESY. All spectra were processed with extensive zero filling in all dimensions in TopSpin 3.1 (Bruker) High-resolution mass spectra were obtained on a MaXis HD QTOF-MS (Bruker Daltronics). The mass spectrometer was optimized to accommodate the larger ions by setting the collision cell parameters: Transfer time to 100 µsec together with collision RF to 1500 Vpp and a pre-pulse storage of 10 µsec. Analytical UHPLC was performed on an Ultima 3000 UHPLC (Dionex) using a 100 × 2 mm, 2.6 µm Kinetex C18 column (Phenomenex). The column was eluted by a linear MeCN-H₂O gradient, containing 20 mM formic acid, from 10 % to 100 % MeCN in 10 min, held at this composition for 2 min before returning to starting conditions at a flow rate of 0.4 mL min⁻¹. All solvents were
purchased from Sigma-Aldrich and were HPLC-grade for extraction and isolation work. For LC-HRMS, all solvents and additives were LC-MS grade.

**Karmitoxin Isolation and Cultivation.** *Karlodinium armiger* (K-0668) was obtained from the Scandinavian Culture Collection for Algae and Protozoa and was cultivated in f/2 media; autoclaved natural seawater (30 PSU) with addition of f/2 vitamins\(^{38}\) and spiked with 50 µM ammonium chloride, kept at 15 °C and an irradiance of 250 µmol photons m\(^{-2}\) s\(^{-1}\). Furthermore, a \(^{13}\)C enriched culture was prepared by the addition of 50 mg·L\(^{-1}\) NaH\(^{13}\)CO\(_3\). As with *K. veneficum*, centrifugation resulted in complete release of toxins to the supernatant. Once the cultures reached stationary phase they were centrifuged by means of continuous centrifugation on a Avanti Series equipped with a JCF-Z rotor (3500 G, 40 mL min\(^{-1}\), Beckman Coulter) and the supernatant loaded directly on C\(_{18}\) Sepra material (Phenomenex) in a SNAP column (60 g, Biotage) that prior had been washed with MeOH and subsequent been equilibrated with milliQ H\(_2\)O. The column was eluted using an Isolera autoflash system (Biotage) by 60 % MeOH, 80 % and 100 % MeOH containing 10 mM formic acid and all fractions were concentrated *in vacuo*. Karmitoxin eluted in 80 % MeOH. Final isolation was achieved by semi-preparative HPLC on a Luna C\(_{18}\)(2) column using a MeCN-H\(_2\)O gradient from 20-50 % MeCN containing 50 ppm TFA over 30 min at a flow rate of 4 mL min\(^{-1}\). From the supernatant of 60 L \(^{13}\)C enriched culture (6.9·10\(^{8}\) cells) we isolated 2.4 mg and from 60 L culture (1.2·10\(^{9}\) cells) we isolated 1.5 mg karmitoxin. At 30% recovery, we calculate a cell toxin quota that ranges from 50 to 100 pg/cell, which is 10 times higher than that found in *K. veneficum* strains.\(^{18}\)

**Karmitoxin (3).** Colorless amorphous solid (1.5 mg); [\(\alpha\)]\(_{25}^{D}\) +17.5 (c 0.08, MeOH); UV (MeCN-H\(_2\)O) \(\lambda\)\(_{\text{max}}\) 226; \(^1\)H and \(^{13}\)C NMR data, see Table 1 and Table S1; HRESIMS \(m/z\) 1386.8875 [M+H]\(^+\) (calcd. for C\(_{73}\)H\(_{127}\)NO\(_{23}\), \(m/z\) 1386.8872, \(\Delta = 0.2\) ppm).

**Karmitoxin Calibration.** Karmitoxin was calibrated by reaction with AccQ Tag and compared to the standard of the derivatized fumonisin-B1. Karmitoxin solution, 10 µL was reacted with 20 µL AccQ tag in 70 µL borate buffer. A calibration curve was prepared by serial dilution, each dilution, 10 µL, by its individual reaction with 20 µL AccQ Tag and 70 µL borate buffer. Analysis was performed on an Ultima 3000 UHPLC (Dionex) equipped with a 1200 series fluorescence detector (Agilent), with fluorescence detection at \(\lambda_{\text{ex}} = 250\) nm and \(\lambda_{\text{em}} = 395\) nm. Separation was achieved on a Kinetex C\(_{18}\) column (Phenomenex, 100x mm, 1.7 µm) using a
MeCN-H₂O gradient containing 50 ppm TFA from 30-100% MeCN in 15 min at a flow rate of 0.400 mL min⁻¹.

**RTgill-W1 Cell Assay.** Purified and calibrated toxins in MeOH (highest MeOH concentration in assay was 1%) were added in 10 concentrations in a serial dilution manner (seven replicates) to a 96 well plate (TPP) containing 20,000 RTgill-W1 cells well⁻¹. The cells had been plated 48 h prior to the experiment in 200 µL well⁻¹ BioWhittaker Leibovitz's L-15 media (Lonza). The gill cells were incubated in the dark at 19 °C for 3 h and subsequently 100 µL of L-15 media with 5 % Presto Blue Cell Viability Reagent (Invitrogen) were added to each well and left for incubation for 30 min.³⁹ The plates were read on a FLUOstar OPTIMA platereader (BMG LABTECH) with λₑₓ = 540 nm and λₑₘ = 590 nm. The viability of the gill cells was calculated as percentage of an L-15 media control. A control plate with MeOH starting at 5 % in L-15 media did not show any cell viability at the highest tested MeOH concentration.

**Stable Isotope Incorporation Experiment** *K. armiger* K-0668 was cultivated with the addition of ¹³C-glycine (Sigma, 99 atom % ¹³C) or ¹⁵N-glycine (Sigma, 98 atom % ¹⁵N) to a final concentration of 1 mM or 0.5 mM ¹³C-glycine (Cambridge Isotopes, 99 atom % ¹³C). The cultures were grown for ~3 generations (10 days) before they were harvested. Extraction was performed by applying 3 mL of each cultures on a Strata-X SPE column (Phenomenex), that previously had been activated with 3 mL MeOH and conditioned with 3 mL Milli-Q H₂O. The column was desalted with 2 mL Milli-Q H₂O and eluted with 1 mL MeOH and subsequent analyzed by LC-HRMS.

**Acartia tonsa Immobilisation and Mortality Assay.** The assay was performed similar to that of Berge et al.²³ in small glass vials in order to avoid absorption of toxin to plastic surfaces. *A. tonsa* were starved 24 h prior to use in the assay. Two adult *A. tonsa* females were added to 2 mL of autoclaved 30 PSU seawater. Karmitoxin was dissolved in MeOH and added in five replicates at seven concentrations. The highest concentration of MeOH was 0.13 %. The blank, in five replicates contained equal amount of MeOH. Immobilization was observed after 2 and 4 h and mortality was noted after 24 h.
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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publication website at DOI:

HRMS spectrum, NMR spectroscopic data, UHPLC-FLD and DP4 analysis.

AUTHOR INFORMATION.

Corresponding Authors

*Tel: (+45) 452-52632. E-mail: tol@bio.dtu.dk and Tel (+45) 353-21985. E-mail: pjhansen@bio.ku.dk

Notes

The authors declare no competing financial interest.

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