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

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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 ¹³C enrichment and high-field 2D NMR spectroscopy, where ¹H–¹³C 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*.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00860. HRMS spectrum, NMR spectroscopic data, UHPLC-FLD, and DP4 analysis (PDF)

The authors declare no competing financial interest.

Marine algae from the cosmopolite dinoflagellate genus *Karlodinium* have been implicated in several fish-killing events.¹ Recently, the responsible fish-killing toxin from *K. veneficum*, named karlotoxin 1 (**1**), was isolated and its structure characterized by van Wagoner et al.² Subsequently, the absolute configuration of karlotoxin 2 (**2**) was assigned in a study by Peng et al.³ 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 and 64 carbon atoms, with two hydroxylated tetrahydropyran rings, one of which has an attached lipophilic arm, while the other has a polyhydroxy arm.⁴ These toxins are related to amphidinols, originally isolated from the dinoflagellate *Amphidinium klebsii*,⁵ compounds that have been shown to exhibit both cytotoxic and antifungal activity.⁶ In addition the amphidinol analogue carteraol E has been shown to be ichthyotoxic, but the experimental conditions were not stated.⁷

Karlotoxins have also been indicated to be produced by *K. conicum*, a recently discovered species from the Australian Ocean. Thus, Mooney et al.⁸ screened several Australian strains of *K. veneficum* and *K. conicum* for hemolytic activity and for their capabilities to produce karlotoxin-like compounds. On the basis of 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 hemolytic activity. In addition, karlotoxins have also been reported in the East China Sea.⁹

The mode of action of karlotoxins has been shown to be related to the disruption of the cell membrane by specific binding to cholesterol,¹⁰ thus creating pores in the membrane similar to that of the amphidinols (e.g. amphidinol 3 (**4**)).¹¹ This behavior has been attributed to the hairpin conformation of this compound class when binding to cholesterol in bilayer membranes.^{11,12} *K. veneficum*'s principle membrane sterol is gymnodinosterol, which renders it immune to its own toxin.¹³ 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.¹⁴

Recently, a new *Karlodinium* species, *K. armiger*, was isolated in the Mediterranean Sea.^{15,16} *K. armiger* has been associated with several reported fish kills and is therefore considered to be an ichthyotoxic species.¹⁶ 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 division per day)¹⁷ on a nitrate-based nutrient medium such

as f/2, unlike *K. veneficum*, which grows well in such nutrient media (0.3 division per day), although better in the presence of prey.¹⁸

Both *K. armiger* and *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.^{19–22} For instance, it has been shown that cultures of *K. armiger* (15 μm) are able to immobilize the much larger copepod *Acartia tonsa* (1 mm) within minutes.²³ Karlotoxins from *K. veneficum* have been shown to be involved in prey immobilization toward copepods, such as *A. tonsa*,^{14,19} that respond by a decreased food uptake.^{20,21} In line with these findings another study reported that whole culture and filtrates of *K. corsicum* cause paralysis of the female copepod *A. grani*.²⁴

In this paper we have identified and characterized a new karlotoxin-like compound, which 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 an RTgill-W1 cell assay²⁵ and toward the copepod *A. tonsa*.

RESULTS AND DISCUSSION

The initial investigation of a *K. armiger* extract identified the presence of a compound with a protonated molecule ($[\text{M} + \text{H}]^+$ of m/z 1386.8875), which combined with the isotopic pattern indicated an elemental composition of $\text{C}_{73}\text{H}_{127}\text{NO}_{23}$. 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 $\text{NaH}^{13}\text{CO}_3$) 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 NH_4^+ should be carefully controlled, due to its equilibrium with the toxic NH_3 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 $\sim 15 \times 10^3$ cells mL^{-1} . By utilizing the ammonium spiking, two large-scale cultivations were carried out, one being in normal natural ^{13}C abundance medium, whereas the other cultivation was performed in the presence of 150 mg L^{-1} $\text{NaH}^{13}\text{CO}_3$. Following cultivation, the compound, hereafter named karmitoxin (**3**), was isolated from the supernatants using standard semipreparative 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, and 15 olefinic protonated carbons, two of which have geminal protons. In addition, the HMBC spectrum and ^{13}C 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.

The structure elucidation was mainly carried out on the ^{13}C -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 ^{13}C -enriched material exhibited $^2J_{\text{CH}}$ 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 $^2J_{\text{CH}}$ 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 correlations 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 by an HMBC correlation from H-44 to C-42. The quaternary carbon C-45 was identified by a long-range $^2J_{\text{CH}}$ 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 (δ_{H} 3.72, δ_{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 ($^3J_{\text{HH}} > 15$ 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 ether linkage between C-37 and C-41 was established by an 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-amino-quinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ Tag). The positioning of the amino group was based on the chemical shift observed for H-1 (δ_{H} 3.54) and the shielded C-1 shift (δ_{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_4H_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 (δ_{H} 1.32) that displayed a large integral in the ^1H NMR spectrum. In addition, the ^{13}C spectrum also revealed several signals around 29 ppm. Hence, we infer that the missing C_4H_8 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

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 tetrahydropyrans 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 ($^3J_{\text{H-40,H-41}} = 12$ Hz and $^3J_{\text{H-39,H-40}} = 12$ 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 $^3J_{\text{H-37,H-38}}$, 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 ($^3J_{\text{H-48,H-49a}} = 12$ Hz and $^3J_{\text{H-49a,H-50}} = 12$ 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 $^3J_{\text{H-51,H-52}}$, suggesting the equatorial orientation of both protons.

Comparison of the ^{13}C and ^1H chemical shifts of the conserved parts of karmitoxin (C-26 to C-54) to those reported for karlotoxin 2 shows almost identical values, except 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 DP4 probability analysis was performed on the polyol arm of new karlotoxin analogues, in order to obtain stereochemical information from experimental and theoretical ^1H and ^{13}C chemical shifts.^{12,27} This method was attempted 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 a very flexible system, see the Supporting Information.

The biosynthesis of the karmitoxin-related amphidinols has been shown to be catalyzed by polyketide synthases by stable isotope incorporation studies using ^{13}C -labeled 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” are 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 1-¹³C-glycine or 1 mM ¹⁵N-glycine for 10 days and 0.5 mM 1,2-¹³C-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 1,2-¹³C-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 using both a rainbow trout gill cell assay (RTgill-W1) and 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 LC₅₀ value of 125 ± 1 nM (Figure 3A). By utilizing fluorescence tagging, similar to what recently has been reported for the derivatization of B-type prymnesins, we observed a similar LC₅₀ value in the RTgill-W1 assay (99 ± 11 nM) (Figure S10).³²

When evaluating the purified karmitoxin toward 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 concentrations (1225–2450 nM). Most of the copepods were dead after exposure to 2450 nM of karmitoxin mL⁻¹ for 4 h, while all copepods were immobilized at a concentration of 1225 nM of karmitoxin mL⁻¹. The 24 h LC₅₀ was calculated to be 400 ± 100 nM (Figure 3B). This is in good agreement with the identified concentration of karmitoxin in culture (288–432 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 mode of action of karlotoxins.^{11,12} In addition, karmitoxin contains a ketone carbonyl also present in karlotoxin-8 and -9^{12,33} and carterao E, lingshuiol, and karatungiols A and B isolated from *Amphidinium* sp.^{7,34,35} Furthermore, it is also noted that karmitoxin has the longest unbranched carbon-carbon chain discovered in this class of compounds, such as the amphidinols^{5,6} and karlotoxins.³³ The length of the lipophilic arm of this class of compounds is important for hemolytic activity,¹² and karmitoxin also exhibits the same length as KmTx1 (C₁₈), which alongside KmTx8 (C₁₆) are the karlotoxins with the highest reported hemolytic activity of rainbow trout erythrocytes.^{12,33} From a toxicity perspective we have shown that karmitoxin is cytotoxic in the mid-nanomolar range in the RTgill-W1 cell assay (Figure 3A), 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^{3,36} 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**).³⁷

EXPERIMENTAL SECTION

General Experimental Procedures.

The optical rotation was measured in MeOH using a PerkinElmer model 241 polarimeter. The UV spectra were 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, and 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 μ s together with collision RF to 1500 Vpp and a prepulse storage of 10 μ s. Analytical UHPLC was performed on an Ultima 3000 UHPLC (Dionex) using a 100 $\times$ 2 mm, 2.6 μ m 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 vitamins³⁸ and spiked with 50 μ M ammonium chloride, kept at 15 °C and an irradiance of 250 μ mol photons m^{−2} s^{−1}. Furthermore, a ¹³C-enriched culture was prepared by the addition of 50 mg L^{−1} NaH¹³CO₃. 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 an Avanti Series centrifuge equipped with a JCF-Z rotor (3500 G, 40 mL min^{−1}, Beckman Coulter), and the supernatant was loaded directly on C₁₈ Septra material (Phenomenex) in a SNAP column (60 g, Biotage) that prior had been washed with MeOH and subsequently been equilibrated with Milli-Q H₂O. The column was eluted using an Isolera autoflash system (Biotage) by 60%, 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 semipreparative HPLC on a Luna C₁₈(2) column using a MeCN–H₂O gradient from 20% to 50% MeCN containing 50 ppm trifluoroacetic acid (TFA) over 30 min at a flow rate of 4 mL min^{−1}. From the supernatant of 60 L of ¹³C-enriched culture (6.9×10^8 cells) we isolated 2.4 mg and from 60 L of culture (1.2×10^9 cells) we isolated 1.5 mg of 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); $[\alpha]^{25}_{\text{D}} +17.5$ (c 0.08, MeOH); UV (MeCN–H₂O); λ_{max} 226; ¹H and ¹³C NMR data, see Table 1 and Table S1; HRESIMS m/z 1386.8875 [M + H]⁺ (calcd for C₇₃H₁₂₇NO₂₃, m/z 1386.8872, δ = 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 of AccQ tag in 70 μL of borate buffer. A calibration curve was prepared by serial dilution, each dilution 10 μL , by its individual reaction with 20 μL of AccQ Tag and 70 μL of borate buffer.

Analysis was performed on an Ultima 3000 UHPLC (Dionex) equipped with a 1200 series fluorescence detector (Agilent), with fluorescence detection at λ_{ex} = 250 nm and λ_{em} = 395 nm. Separation was achieved on a Kinetex C₁₈ column (Phenomenex, 100x mm, 1.7 μm) using a MeCN–H₂O gradient containing 50 ppm TFA from 30% to 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) was added to each well and left for incubation for 30 min.³⁹ The plates were read on a FLUOstar OPTIMA plate reader (BMG LABTECH) with λ_{ex} = 540 nm and λ_{em} = 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 1-¹³C-glycine (Sigma, 99 atom % ¹³C) or ¹⁵N-glycine (Sigma, 98 atom % ¹⁵N) to a final concentration of 1 or 0.5 mM 1,2-¹³C-glycine (Cambridge Isotopes, 99 atom % ¹³C). The cultures were grown for about three generations (10 days) before they were harvested. Extraction was performed by applying 3 mL of each culture on a Strata-X SPE column (Phenomenex), which previously had been activated with 3 mL of MeOH and conditioned with 3 mL of Milli-Q H₂O. The column was desalted with 2 mL of Milli-QH₂O, eluted with 1 mL of MeOH, and subsequently analyzed by LC-HRMS.

Acartia tonsa Immobilization and Mortality Assay.

The assay was performed similarly 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of the elucidated backbone of karmitoxin, where bold red bonds illustrate the parts that were assembled by long-range $^2J_{CH}$ correlation and blue bold bonds show the parts assembled by COSY/TOCSY correlations, together with key HMBC correlations connecting the different parts.

Figure 2.
Relative configuration of the two pyran rings of karmitoxin.

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 be 125 ± 1 nM. Dose-reponse 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 be 400 ± 100 nM.

Chart 1.

Table 1.

NMR Spectroscopic Data (¹H 800 MHz, ¹³C 200 MHz CD₃OD) for Karmitoxin (3)^a

pos	δ _C	δ _H (J in Hz)	pos	δ _C	δ _H (J in Hz)	pos	δ _C	δ _H (J in Hz)
1	40.8, CH ₂	3.56	26	29.8, CH ₂	2.14, m	49	29.9, CH ₂	1.57, m
2	123.8, CH	5.74, m (15.1)	27	73.0, CH	3.64, dd (8.9, 3)			2.11, m
3	135.5, CH	6.42, dd (15.1, 10.5)	28	72.5, CH	3.32, m	50	65.7, CH	4.07, m
4	128.3, CH	6.28, m (15.4, 10.5)	29	70.0, CH	3.86, m	51	67.1, CH	4.06, m
5	138.4, CH	5.87, dd (15.4, 6.1)	30	33.8, CH ₂	1.51, m	52	79.2, CH	3.74, m
6	71.3, CH	4.14, m			1.68, m	53	70.3, CH	4.00, m
7	40.2, CH ₂	2.21, m	31	22.9, CH ₂	1.45, m	54	72.7, CH	4.38, dd (9.4, 3.1)
		2.25, m			1.53, m	55	127.0, CH	5.61, dd (15.2, 7.6)
8	126.0, CH	5.48, m (16)	32	31.6, CH ₂	1.28, m	56	134.6, CH	5.81, m
9	132.5, CH	5.51, m (16)			1.69, m	57	32.3, CH ₂	2.11, m
10	28.3, CH ₂	2.10, m	33	34.9, CH ₂	1.75, m	58	29.0, CH ₂	1.43, m
		2.15, m	34	78.5, CH	3.11, m	59	29.0, CH ₂	1.32, m
11	36.5, CH ₂	1.51, m	35	67.0, CH	3.90, m	60	29.0, CH ₂	1.32, m
12	66.5, CH	4.06, m	36	33.0, CH ₂	1.69, m	61	29.0, CH ₂	1.32, m
13	50.5, CH ₂	2.61, m			1.84, m	62	29.0, CH ₂	1.32, m
14	210.2, C		37	73.1, CH	4.11, m	63	29.0, CH ₂	1.32, m
15	50.4, CH ₂	2.61, m				64	29.0, CH ₂	1.32, m
16	67.2, CH	4.07, m	38	71.6, CH	3.72, m	65	29.0, CH ₂	1.41, m
17	36.9, CH ₂	1.41, m	39	71.6, CH	3.72, m	66	32.3, CH ₂	2.10, m
		1.52, m	40	69.4, CH	3.86, m	67	134.9, CH	5.72, m (15)
18	21.3, CH ₂	1.40, m	41	76.6, CH	3.40, m	68	131.0, CH	6.06, dd (15, 10)
		1.62, m	42	70.9, CH	4.03, m	69	137.3, CH	6.32, m (10)
19	36.0, CH ₂	1.42, m	43	29.9, CH ₂	1.70, m	70	113.5, CH ₂	4.95, dd
		1.55, m			2.03, m			5.05, dd
20	70.9, CH	3.61, m	44	26.0, CH ₂	2.13, m			
21	40.0, CH ₂	2.21			2.45, m	71	11.1, CH ₃	0.92, m

pos	δ_C	δ_H (J in Hz)	pos	δ_C	δ_H (J in Hz)	pos	δ_C	δ_H (J in Hz)	pos	δ_C	δ_H (J in Hz)
22	126.9, CH	5.70, m (16, 8.6)	45	151.0, C		72	15.3, CH ₃	0.98, d (7.0)			
23	136.0, CH	5.55, (16, 6.6)	46	75.5, CH	4.23, d (8.9)	73	112.0, CH ₂	5.03, s			
24	70.1, CH	4.16, m	47	73.4, CH	3.38, m			5.08, s			
25	41.7, CH ₂	1.45, m 1.65, m	48	68.8, CH	4.06, m						

^a All signals are referenced to residual CH₃OH at δ_H 3.31 ppm and δ_C 47.1 ppm. Coupling constants in congested regions were extracted from the phase-sensitive DQF-COSY data. HMBC correlations are shown in Table S1.