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Two new karlotoxins found in *Karlodinium veneficum* (strain GM2) from the East China Sea

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Abstract

The dinoflagellate *Karlodinium veneficum* is a harmful algal bloom species with a worldwide distribution. This small athecate dinoflagellate makes a family of polyketide toxins that are hemolytic, cytotoxic and ichthyotoxic. The first chemical structure for karlotoxins from East China Sea (ECS) is reported here. The two new karlotoxins, namely 4,5-dihydro-KmTx 2 (compound **1**) and 4,5-dihydro-dechloro-KmTx 2 (compound **2**), were isolated and purified from monoalgal cultures of *K. veneficum* strain GM2. Their structures were determined by spectroscopic analysis, including tandem mass spectrometry as well as 1D and 2D NMR experiments. These new karlotoxin congeners feature a saturated polyol arm different from previously reported for KmTx 2 that appears to increase hemolytic activity.

Keywords

Dinoflagellate; *Karlodinium veneficum*; Karlotoxin; East China Sea; hemolysis

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1. Introduction

For more than half a century, the dinoflagellate *Karlodinium veneficum* (aka *Gymnodinium veneficum*) has been associated with fish kill incidents worldwide (Abbot and Ballantine, 1957; Braarud, 1957, reviewed in Place et al. 2012). It is frequently present in relatively low cell abundance ($10^2 - 10^3$ cells·mL⁻¹), but is capable of forming intense blooms of $10^4 - 10^5$ cells·mL⁻¹ that could cause severe aquatic faunal mortalities (Deeds et al. 2002; Kempton et al. 2002; Adolf et al., 2008). *K. veneficum* is a mixotrophic microalgae, living on both photosynthesis and phagotrophy (; Adolf et al., 2006b; Calbet et al., 2011). *K. veneficum* produces a unique suite of polyketide toxins called karlotoxins or KmTxs, with hemolytic, cytotoxic, and ichthyotoxic properties (Bachvaroff et al., 2008). In nature, karlotoxins function as anti-grazing or allelopathic compounds (Adolf et al., 2007; Waggett et al., 2008) as well as prey immobilization agents for mixotrophy (Sheng et al., 2010). It wasn't until 2008 that the first planar structure of karlotoxin was reported (Van Wagoner et al., 2008) with the absolute configuration appearing two years later (Peng et al. 2010). Up to now, structures of seven karlotoxins have been reported as well as some tentatively assigned ones (Place et al., 2012). They feature a complex hairpin-like structure with three distinct regions: a polyol arm bearing variable hydroxylation and methylation; a hinge region containing two pyran rings; and a lipophilic arm with a terminal diene (Place et al., 2012). The unique structure enables karlotoxins to form pores in cell membranes leading to cell lysis through colloid osmolysis (Deeds et al., 2006). During pore formation, karlotoxins selectively bind 4-desmethyl sterols in cell membranes (Adolf et al. 2006a; Deeds and Place, 2006) making them important marine drug candidates for the treatment of severe human health issues including heart disease and cancer where cholesterol is involved (Waters et al., 2010; Waters et al. 2015).

Since the first description of *K. veneficum* in East China Sea, ECS (Zhou et al., 2008), it has been repeatedly identified in this area (Wang et al., 2011; Dai et al., 2014). It may pose potential threats in ECS, both in open water and nearby aquaculture areas (Zhou et al., 2011). Instead of forming single-species bloom, *K. veneficum* frequently co-occurred with other harmful algae, such as *Karenia mikimotoi* and *Prorocentrum donghaiense*, as an ECS red tide (Zhou et al., 2008), however, whether this species is endemic or was imported by ballast water is still unknown. In our previous study, *K. veneficum* may terminate *P. donghaiense* blooms through grazing, and initiate its own subsequent blooms in ECS (Zhou et al., 2015). That the toxic strain GM2 from ECS may produce unique karlotoxin congeners was revealed by LC/MS data (Zhou et al., 2015). The structure of karlotoxin from ECS strains has not yet been characterized. Here, the isolation and structural elucidation of two new karlotoxins from this strain are presented (Fig. 1), and their hemolytic potential relative to KmTx 2 are characterized. The new karlotoxins and KmTx 2 have the same carbon skeleton, and the new karlotoxins will be referred to as “compound 1” and “compound 2” in the following text.

2. Materials and methods

2.1 General experimental procedures

Optical rotations were acquired on a JASCO P-2000 Digital polarimeter. UV spectra were measured using a Thermo Evolution 201 spectrophotometer. All NMR spectra were acquired in 1:2 pyridine-d₅/MeOH-d₄ on a 600 MHz Agilent DD2 spectrometer with a 5 mm OneNMR probe. NMR data were analyzed using MestReNova 8.1. High-resolution mass spectra (HRMS) were obtained using a Bruker Apex FT-ICR mass spectrometer with an ESI interface (compound **1**) and a Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer with HCCA matrix (compound **2**). MS/MS experiments were conducted on an AB SCIEX 4000 QTRAP mass spectrometer, with a collision energy of 35 eV. Semi-preparative HPLC was accomplished using a system with two Waters 515 HPLC pumps, a gradient controller, and a Waters 2996 diode array UV detector.

2.2 Hemolytic assay

Erythrocytes were extracted from the caudal vein of gilthead seabream (*Sparus aurata*), and the blood washed three times with three volumes of buffer per mL of packed erythrocytes using the incubation buffer described below minus CaCl₂. Following the third wash, the cells were resuspended in 3 mL of buffer per mL of packed cells. The resuspended cells were diluted 1:20 in buffer (pH 7.5; 150 mM NaCl, 3.2 mM KCl, 1.25 mM MgSO₄, 12.2 mM Tris Base, and 3.75 mM CaCl₂) and 200 µL of diluted, washed erythrocytes were incubated for 1 hour at 25 °C in the presence of purified toxins serially diluted in methanol for testing. After incubation, remaining intact erythrocytes were pelleted by centrifugation, and the OD at 540 nm was read using a Molecular Devices Vmax (Sunnyvale, CA, USA) microtiter plate spectrophotometer. Saponin (0 to 20 µg) (from Quillaja bark; Sigma-Aldrich, S-4521, St Louis, MO, USA) was used as a positive control. The dose response curves [Fractional Hemolysis vs. log (µg mL⁻¹ of karlotoxin)] were fitted to the Hill equation:

$$\text{Fractional Hemolysis} = \text{Base Hemolysis} + (\text{Maximal Hemolysis} - \text{Base Hemolysis}) / [1 + (\text{Toxin giving 50\% hemolysis})^{\text{Rate of Hemolysis}}]$$

using a nonlinear regression (Igor 6.02, Wavemetrics, Lake Oswego, OR, USA). In all cases, convergence to defined parameters was observed.

Known aliquots of the purified toxins were placed onto a tared aluminum weigh boat in triplicate, dried at 60°C overnight, and weighed the following day on a Mettler UMT2 microgram balance (Mettler, 900 Polaris Parkway, Columbus, OH, USA). Purified toxins were also serially diluted in methanol for testing. Known dilutions of toxin were then mixed with water (25 µL toxin + 50 µL water), and 5, 10, and 50 µL injections of each sample were run in triplicate to determine the UV and MS response factors of the material tested.

2.3 Cancer cytotoxicity assay

THP-1 human monocytic leukemia cells from CCTCC (Wuhan, China) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2% glutamine, 100 U/mL

of penicillin, and 100 µg/mL of streptomycin at 37 °C with 5% CO₂. The MTT (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyltetrazolium bromide) cytotoxicity assays were performed for the 24 h cultured THP-1 cells treated with the karlotoxins to determine the cellular survival. Absorbance was measured at a wavelength of 492 nm. All experiments were repeated three times, and the data were presented as the IC₅₀ of the two new karlotoxins.

2.4 Biological material

The dinoflagellate *Karlodinium veneficum* strain GM2 (NMB jah047-1 in Microalgae Collection in Ningbo University) was originally isolated from a seawater sample collected in Yushan, East China Sea (ECS), in June 2006 (Zhou et al., 2015). The strain was previously under the taxonomic synonym '*Karlodinium micrum*'. Cultures were grown in 5 L batches (200 L total) using natural seawater from the coastal ECS 23~25 PSU (practical salinity units), which was filtered with 0.45 mm acetate cellulose membrane (Millipore, USA), and sterilized by boiling for 3 min. The culture media was f/2-Si. Cultures were grown using a 12/12 h light/dark cycle at 23 °C.

2.5 Toxin isolation

The cultures were harvested using GF/F filters (Whatman, Inc.). The filtrate was loaded onto a RP column (3 × 20 cm) using YMC ODS-C18 (50 µm), and eluted with a step gradient consisting of 1000 mL of 30% aqueous MeOH, then 100 mL each of 60%, 80% aqueous MeOH and 100% MeOH. The karlotoxins eluted at 80% MeOH fraction, which was further purified by semi-preparative HPLC using a YMC C18 column (10 × 250 mm; 5 µm) and isocratic elution with 75% aqueous MeOH at 2 mL/min to yield compound **1** and compound **2**.

3. Results

3.1 Isolation and purification of karlotoxin

When cultures of *K. veneficum* are harvested by filtration or centrifugation, the karlotoxins are readily released from the algal cells (Bachvaroff et al. 2008). The subsequent filtrate or supernatant can be absorbed onto RP-C18 material, washed with deionized distill water to desalt and then eluted with increasing organic solvents to recover the karlotoxins. Following elution with 5 to 10 column volumes of 30% MeOH to remove additional salt and pigments, HPLC analysis of the 80% MeOH fraction showed two major peaks with typical karlotoxin UV characteristics. Further purification on a C18 semi-preparative HPLC (Fig. 2) yielded compound **1** (11.8 mg) and compound **2** (5.2 mg).

3.2 Structure elucidation of compound 1

Compound **1** was obtained as a colorless amorphous powder; $[\alpha]_D^{25} +8$ (c 0.002, MeOH). The UV absorption at 235 nm (MeOH, ϵ 4.1) indicated the presence of a terminal chlorodiene structure. HRMS of compound **1** suggested a molecular formula of C₆₇H₁₂₃ClO₂₄ (m/z [M + H]⁺ 1347.8136 (calculated for C₆₇H₁₂₄ClO₂₄, 1347.8171; δ = -2.6 ppm)), with two additional protons when compared to that of KmTx 2 (C₆₇H₁₂₁ClO₂₄) (Peng et al., 2010). The molecular formula was supported by the ¹H and ¹³C NMR spectra, the latter of which revealed 67 signals, which were assigned to one quaternary sp² carbon, six sp²

methines, one sp^2 methylene, 25 oxygenated methines, one oxygenated methylene, three aliphatic methines, 27 aliphatic methylenes, and three aliphatic methyls (Table 1). This differs from a similar count for KmTx 2 by loss of two sp^2 methines and two additional aliphatic methylenes, suggesting that one of the five double bonds in KmTx 2 was saturated in compound **1**. Comparison of the 1D and 2D NMR spectra of compound **1** and KmTx 2 showed them to be nearly identical to each other, while all of the structural features of KmTx 2 are present in compound **1**, except the C4–C5 vinyl group in the polyol arm. Substructure C1–C7 was deduced directly from the correlated spectroscopy (COSY) data and heteronuclear multiple bond correlation (HMBC) (Fig. 3), which confirmed the absence of a C4–C5 double bond. Further support was provided by comparison of the mass spectrum resulting from collisionally induced dissociation (CID) of compound **1** to that of KmTx 1 bearing the C4–C5 vinyl group (Van Wagoner et al., 2008). The MS/MS spectrum of compound **1** showed serial fragmentation ion peaks at 1113, 939, 735, and 677 (Fig. 4), all of which are 2 Da higher than the corresponding fragments in KmTx 2 (Bachvaroff et al. 2008, Van Wagoner et al., 2008). Furthermore, the locations of two ether rings and geometries of the double bonds agreed with those of other karlotoxins. Thus, the planar structure of compound **1** was identified as 4,5-dihydro-KmTx 2 as shown in Fig. 1, which was a new member of the karlotoxin family representing the first occurrence of karlotoxin with a saturated polyol arm.

3.3 Structure elucidation of compound 2

Compound **2** was obtained as a colorless amorphous powder; $[\alpha]_D^{25} +11$ (c 0.0015, MeOH). The UV absorption at 225 nm (MeOH, ϵ 4.1) indicated that compound **2** contained a terminal diene without chlorine. The molecular formula of compound **2** was determined by HRMS as $C_{67}H_{124}O_{24}$ (m/z $[M + Na]^+$ 1335.8401 (calculated for $C_{67}H_{124}O_{24}Na$, 1335.8380; $\delta = 1.6$ ppm)). A comparison between the molecular formulae of compound **1** and compound **2** revealed that compound **2** was the dechloro form of compound **1**. The ^{13}C NMR of compound **2** was nearly identical to compound **1** except for the absence of a sp^2 methine and an additional sp^2 methylene at δ 115.16 (Table 1), the latter of which showed a negative signal in DEPT 135° spectrum. This assignment was supported by an additional vinyl proton in the 1H NMR and HSQC spectra of compound **2**, and further corroborated by serial coupling from H-57 to H-63 in its COSY spectrum. The only difference between compound **1** and compound **2** was at the lipophilic arm, which coincided with the fact that the MS/MS fragmentation pattern of compound **2** was identical to that of compound **1** (Fig. 4). Detailed analysis of 1D and 2D NMR data enabled assignment of most key signals, although some methylenes and hydroxy methines remained unassigned due to signal overlapping (Table 1). Consequently, the planar structure of compound **2** was elucidated as another new karlotoxin, 4,5-dihydro-dechloro-KmTx 2 (Fig. 1).

3.4. Biological activities of the two new karlotoxins

These two new karlotoxins show strong hemolytic activity against erythrocytes extracted from the caudal vein of gilthead seabream (*Sparus aurata*) (Figure 5), with IC_{50} at 997 ± 37 ng/ml and 943 ± 39 ng/ml, for compound **1** and compound **2**, respectively. This is nearly 2 fold more potent than KmTx 2. The cytotoxicity of compound **1** and compound **2** against the

THP-1 human monocytic leukemia cell line was with IC₅₀ at 15 μ M and 36 μ M, respectively.

4. Discussion

Seven karlotoxins have been isolated and structurally characterized from two *K. veneficum* strains of American origin (CCMP 2064 and CCMP 2936) (Place et al., 2012). The present paper reports the first isolation of new karlotoxin analogs from *K. veneficum* collected in ECS. As is clear in the present report the toxin profile of *K. veneficum* differs between strains as seen previously (Bachvaroff et al. 2009), similar to a phenomenon observed in other toxigenic dinoflagellates, e.g. amphidinols from *Amphidinium* spp (Echigoya et al., 2005). Considering the wide distribution of *K. veneficum*, it is not unexpected that strains from different locations may produce toxins with different chemical structures and toxicities.

Instead of using the wavelength of maximum UV absorption as a means for karlotoxin classification (Kempton et al., 2002), now the congeners are classified according to the length of the lipophilic side chain. There are currently three groups of karlotoxins, namely KmTx 1, 2, 3 with 18, 17, and 16 carbons in length, respectively. Compound **1** and compound **2** both contain a side chain of 16 carbons (C-48 to C-63). Thus, they belonged to the KmTx 2 group, and are named after their parent molecule. The most distinctive feature of karlotoxin isolated from ECS was the saturated polyol arm not previously described. Although the biosynthetic pathway leading to this structural feature is unknown at this time, an additional enoyl reductase module of the polyketide synthase is suspected to be involved. The addition of a saturated polyol to KmTx 2 structure appears to confer greater hemolytic toxicity lowering the HD50% by a factor of 2 (Fig. 5). In a recent investigation on the mechanism of action, Waters et al. (2015) showed that KmTx binds to a sterol on the cell membrane, and inserts the polyol arm into it, to form a pore on the cell membrane with multiple molecules in a micelle-like interaction. The saturated polyol arm may have better structural flexibility, which would promote the pore formation process. Furthermore, there appears to be no statistical difference between compound **1** and compound **2** in hemolytic potential, suggesting that chlorine functionality does not contribute to the hemolytic activity. In addition, compound **1** and compound **2** were cytotoxic against the THP-1 human monocytic leukemia cell line, with IC₅₀ at 15 and 36 μ M, respectively, which merits further investigation into their anti-tumor potential.

Owing to the complexity of karlotoxin's structure, only the absolute configuration of KmTx 2 has been determined by chemical degradation work (Peng et al., 2010). The stereochemistry of compound **1** and compound **2** still awaits future investigation.

5. Conclusions

The present paper reports the discovery of two new karlotoxins from *K. veneficum* strain GM2 collected in ECS, and brings the total number of natural karlotoxin analogs to nine at this time. Karlotoxins from ECS featured a saturated polyol which differs from toxins of American origin. Both new toxins show greater hemolytic activities than KmTx 2. This study provides new insight into the toxic effect caused by *K. veneficum* in ECS. The new

toxins can now be employed as standards for rapid detection of karlotoxin in ECS water using LC/MS with selective ion monitoring, which may help detect and manage toxic *K. veneficum* blooms in this area. Having purified standards for site specific karlotoxins has been extremely valuable in monitoring fishkills in US (Bachvaroff et al. 2008) and Australia (Adolf et al. 2015). The Chinese local marine environment department and fishery department may use these new karlotoxins to monitor sea water from coastal area and aquaculture pond. With their potent biological activities and sustainable sample supply, they may also serve as new targets for marine drug discovery.

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Fig. 1.
Chemical structures of compound **1** (4,5-dihydro-KmTx 2) and compound **2** (4,5-dihydro-dechloro-KmTx 2).

Fig. 2.

Typical reverse phase HPLC chromatogram for the isolation of compound **1** (4,5-dihydro-KmTx 2) and compound **2** (4,5-dihydro-dechloro-KmTx 2). The UV spectra of both peaks are shown as inset.

Fig. 3.

Structural elucidation by detailed analysis of 2D NMR data, including key HMBC (indicated by arrows from ^1H to ^{13}C) and ^1H - ^1H COSY (indicated by bold lines) for compound **1** and compound **2**.

Fig. 4. (A) MS/MS spectrum of compound **1**, which was similar with that of compound **2**. (B) Elucidation of fragmentation pattern by tandem MS/MS of $[\text{M}+\text{Na}]^+$. All m/z values correspond to ion fragments containing sodium ions.

Fig. 5.

Comparative hemolytic potency for compound **1** & compound **2** compared to KmTx 2 and saponin using Gilthead seabream (*Sparus aurata*) erythrocytes. Note the logarithmic scale for toxin amount. The fitted line is based on the Hill equation, and the HD50% estimates for these curves are presented in the legend to the figure.

Table 1

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

position	1		2	
	δ_C , mult.	δ_H , mult, (J in Hz)	δ_C , mult.	δ_H , mult, (J in Hz)
1	67.48, CH ₂	3.55, dd (12.2, 5.5); 3.59, dd (11.0, 4.6)	67.49, CH ₂	3.55, dd (12.1, 5.5); 3.58, dd (11.0, 4.6)
2	73.15, CH	3.71, m	73.16, CH	3.71, m
3	34.65, CH ₂	1.56, m; 1.61, m	34.66, CH ₂	1.55, m; 1.61, m
4	22.96, CH ₂	1.43, m	22.97, CH ₂	1.44, m
5	34.90, CH ₂	1.45, m	34.91, CH ₂	1.45, m
6	72.01, CH	3.59, m	72.01, CH	3.59, m
7	33.12, CH ₂	1.65, m; 1.74, m	33.13, CH ₂	1.65, m; 1.74, m
8	22.76, CH ₂	1.44, m	22.77, CH ₂	1.45, m
9	36.45, CH ₂	1.78, m	36.46, CH ₂	1.78, m
10	74.00, CH	3.50, m	74.01, CH	3.50, m
11	38.90, CH ₂	1.47, m	38.90, CH ₂	1.47, m
12	23.85, CH ₂	1.44 ^b , m	23.85, CH ₂	1.44 ^b , m
13	39.14, CH ₂	1.45 ^b , m	39.14, CH ₂	1.45 ^b , m
14	70.52, CH	4.24, m	70.53 ^b , CH	4.24, m
15	45.04, CH ₂	1.61, m	45.04, CH ₂	1.61, m
16	33.12, CH	1.96, m	33.13, CH	1.96, m
17	79.92, CH	3.14, dd (6.9, 3.0)	79.93, CH	3.14, dd (7.0, 3.3)
18	72.72, CH	3.68, m	72.72, CH	3.68, m
19	31.82, CH ₂	1.63, m; 1.73 ^b , m	31.82, CH ₂	1.64, m; 1.73 ^b , m
20	31.48, CH ₂	1.45, m; 1.73 ^b , m	31.50, CH ₂	1.45, m; 1.72 ^b , m
21	35.20, CH	2.03, m	35.2, CH	2.03, m
22	74.00, CH	3.88, d (10.3)	74.01, CH	3.88, d (10.1)
23	74.00, CH	3.50, d (9.0)	74.01, CH	3.50, d (9.0)
24	71.72, CH	4.05, m	71.72, CH	4.04, m
25	35.43, CH ₂	1.61, m; 1.81, m	35.44, CH ₂	1.61, m; 1.81, m
26	24.45, CH ₂	1.46 ^b , m; 1.53, m	24.46, CH ₂	1.45 ^b , m; 1.52, m
27	33.08, CH ₂	1.27, m; 1.72 ^b , m	33.08, CH ₂	1.27, m; 1.72 ^b , m
28	36.57, CH	1.78, m	36.58, CH	1.78, m
29	78.94, CH	3.17, dd (7.0, 3.2)	78.95, CH	3.17, dd (7.1, 3.3)
30	68.40, CH	4.04, m	68.40, CH	4.05, m
31	34.90, CH ₂	1.81, m; 1.94, m	34.91, CH ₂	1.81, m; 1.94, m
32	74.80, CH	4.36, dd (11.0, 3.0)	74.66, CH	4.36, dd (11.1, 3.0)
33	73.15, CH	3.90, dd (2.9, 2.9)	73.16, CH	3.90, dd (3.2, 3.2)
34	73.15, CH	3.94, dd (7.8, 3.1)	73.16, CH	3.93, dd (7.8, 3.1)
35	71.38, CH	4.14, dd (8.0, 8.0)	71.38, CH	4.14, dd (7.9, 7.7)
36	78.01, CH	3.58, m	78.06, CH	3.58, m

position	1		2	
	δ_C , mult.	δ_H , mult, (J in Hz)	δ_C , mult.	δ_H , mult, (J in Hz)
37	72.08, CH	4.20, m	72.09, CH	4.21, m
38	31.99, CH ₂	1.83, m; 2.19, m	31.96, CH ₂	1.83, m; 2.19, m
39	27.57, CH ₂	2.26, m; 2.65, m	27.56, CH ₂	2.26, m; 2.65, m
40	150.05, C		151.85, C	
41	76.85, CH	4.41, d (8.8)	76.89, CH	4.41, d (8.8)
42	75.06, CH	3.53, m	75.08, CH	3.53, m
43	70.52, CH	4.22, m	70.54, CH	4.22, m
44	31.88, CH ₂	1.64, m; 2.34, m	31.90, CH ₂	1.64, m; 2.34, m
45	67.48, CH	4.20, m	67.49, CH	4.18, m
46	68.70, CH	4.34, m	68.70, CH	4.34, m
47	80.65, CH	4.00, d (10.0)	80.67, CH	4.00, d (10.0)
48	72.59, CH	4.20, m	72.60, CH	4.19, m
49	74.18, CH	4.62, dd (7.1, 2.2)	74.19, CH	4.62, dd (7.4, 2.9)
50	129.07, CH	5.75, dd (15.5, 7.2)	129.08, CH	5.75, dd (15.2, 7.3)
51	135.27, CH	5.82, dt (13.3, 6.7)	135.17, CH	5.82, dt (13.5, 6.8)
52	33.50, CH ₂	1.95, m	33.54, CH ₂	1.95, m
53	30.14, CH ₂	1.26, m	30.21, CH ₂	1.26, m
54	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m
55	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m
56	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m
57	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m	29.40-31.17 ^b , CH ₂	1.12-1.20 ^b , m
58	30.19, CH ₂	1.27, m	30.17, CH ₂	1.27, m
59	33.52, CH ₂	1.97, m	33.44, CH ₂	1.98, m
60	137.20, CH	5.66, dt (14.7, 7.0)	136.28, CH	5.64, dt (14.7, 7.0)
61	127.83, CH	5.98, dd (15.1, 10.9)	132.23, CH	6.01, dd (15.2, 10.4)
62	135.15, CH	6.40, dd (12.9, 11.0)	138.57, CH	6.28, dd (17.0, 10.2)
63	119.38, CH	6.21, d (13.1)	115.16, CH ₂	5.04, d (17.0); 4.89, d (10.2)
64	16.91, CH ₃	0.94, d (6.4)	16.91, CH ₃	0.94, d (6.4)
65	13.49, CH ₃	0.99, d (6.7)	13.49, CH ₃	0.99, d (6.7)
66	16.86, CH ₃	0.92, d (6.5)	16.87, CH ₃	0.92, d (6.6)
67	112.97, CH ₂	5.01, s; 5.13, s	112.98, CH ₂	5.01, s; 5.14, s

^aChemical shifts referenced to residual methanol-*d*⁴ signals at 3.31 and 49.0 ppm for ¹H and ¹³C, respectively.

^bUncertain assignment due to signal overlap.