Two new eunicellin diterpenoids from the South China Sea gorgonian
*Muricella sibogae* and their bioactivities

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A systematic re-study on gorgonian *Muricella sibogae* from South China Sea yielded 10 eunicellin-based diterpenoids including two new ones, sibogins C and D (1 and 2). Their structures were elucidated by extensive spectroscopic analyses (1D and 2D NMR, IR and MS) and by comparison with data reported in literatures. All the isolates were tested for cytotoxic and antifouling activities. Compounds 3 and 5 showed significant antifouling activity against the green mussel *Perna viridis*, and especially 3 was suggested as a potent low-toxic antifouling agent with a large LC\(_{50}/\)EC\(_{50}\) value of 18.6. This was the first report on the antifouling activity of the eunicellin-type diterpenoids against the green mussel.

**Keywords:** gorgonian; *Muricella sibogae*; eunicellin-based diterpenoids; cytotoxicity; antifouling activity

1. Introduction

Marine gorgonians (order Gorgonacea, subclass Octocorals, phylum Cnidaria) have been the abundant origin of unique structure and bioactive secondary metabolites (Blunt et al. 2012; Blunt et al. 2013). There were about 80 species of gorgonian corals from seven families reported in China (Fu et al. 2009), in which the genus *Muricella* (family Paramuriceidae) was characterised by eunicellin-based diterpenoids (Seo et al. 1997, 2000) with a cladiellane skeleton containing an ether bridge across C-2 and C-9. These metabolites displayed a wide range of bioactivities, such as cytotoxicity (Seo et al. 1997, 2000), lethality toward brine shrimp (Seo et al. 1997) and antifouling activity (Lai et al. 2012). As a rarely examined species, *Muricella sibogae*

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was firstly found to have eunicellin diterpenoids in 2013 from our previous study (Li et al. 2013). In order to further study the diterpenoid structural diversity and bioactivity, we re-collected this specimen from Weizhou Island in 2010. Structure-guided chemical investigation on *M. sibogae* resulted in the isolation of ten eunicellin-based diterpenoids (Figure 1), including two new analogues, sibogins C and D (1 and 2). All the isolates were evaluated for cytotoxic and antifouling activities against selected models. Compounds 3 and 5 showed potential inhibitory activity on the settlement of the green mussel *Perna viridis*. Here, we reported the isolation, structure elucidation and bioactivity of the compounds.

2. Results and discussion

Compound 1 was isolated as a colourless oil. Its molecular formula was established as C\textsubscript{23}H\textsubscript{36}O\textsubscript{4} by the HR-ESI-MS ion peak [M + Na]\textsuperscript{+} at m/z 399.2504, requiring six degrees of unsaturation. The IR spectrum absorbed at 1733 cm\textsuperscript{-1} revealed the presence of carbonyl group. In the \textsuperscript{13}C NMR spectrum, a total of 20 skeleton-carbon signals, except for an OMe (\(d_C\) 55.5) and an OAc (\(d_C\) 169.6, 23.0) groups, indicated that 1 could be a diterpenoid. Four olefinic carbon resonances at \(d_C\) 137.1, 129.8, 126.1 and 121.9 indicated the fact of two double bonds. Thus, 1 was rationally speculated to have a tricyclic skeleton. Together with the characteristic proton signals, two oxygenated CH groups (\(d\) 4.42 and 4.20), three relative downfield singlet Me groups (\(d\) 1.79, 1.75 and 1.72) and an i-Pr group (\(d\) 1.61, 0.99 and 0.91, from \textsuperscript{1}H–\textsuperscript{1}H COSY spectrum), especially the key HMBC correlation from H-2 (\(d\) 4.42, 1H, \(d, J = 6.6\) Hz) to C-9 (\(d\) 81.8) indicative of the presence of the ether bridge across C-2 and C-9, suggested 1 to be a eunicellin-type diterpenoid. Comparison of the 1D NMR data of 1 with those of 4, a co-isolated known compound, revealed their closely structural similarity, except for an extra MeO group (\(d_H\) 3.29 and \(d_C\) 55.5) in 1, which was supported by the observation of 30 mass units more than 4 and the presence of an additional oxygenated methine signal (\(d_H\) 3.59, br s, H-13; \(d_C\) 76.1, C-13) in 1. The consecutive \textsuperscript{1}H–\textsuperscript{1}H COSY correlations from H-12 to H-2 (Supplementary material Figure S1), and the crucial HMBC correlation of H\textsubscript{3}–OCH\textsubscript{3} (\(d_H\) 3.29) with C-13 (\(d_C\) 76.1) could

![Figure 1. Eunicellin diterpenoids from gorgonian *Muricella sibogae*.](image)
position the OCH$_3$ at C-13. Assignation of OAc group at C-3 was evident from the remarkable downfield chemical shift of C-3 ($\delta_C$ 89.6). Finally, the planar structure of 1 was established by careful 2D NMR spectral explanation, especially the HMBC correlations of H$_3$-19 or H$_3$-20 with C-14, H-2 with C-9, H$_3$-15 with C-2, C-3 and C-4, H$_3$-16 with C-6, C-7 and C-8, H$_3$-17 with C-10, C-11 and C-12 (Supplementary material Figure 1).

The relative configurations of 1 were determined by the NOESY experiment and coupling constant calculation. NOE correlations of H-2 (δ$_H$ 4.42) with H-9 (δ$_H$ 4.20), H-14 (δ$_H$ 1.92), and Me-15 (δ$_H$ 1.72) showed their cis relations arbitrarily assigned α-orientations. Additional NOE interactions from H$_3$-19 and H$_3$-20 (δ$_H$ 0.99 and 0.91) to H-1 (δ$_H$ 2.44), H-10 (δ$_H$ 2.40, br d, $J = 8.5$ Hz) and H-13 indicated the β-orientations of H-1, H-10, H-13, and r-Pr. The olefinic geometries was determined to be 6E and 11Z according to the NOE cross peaks between H$_3$-16 (δ$_H$ 1.79) and H-5 (δ$_H$ 2.36, 2.13), and between H$_3$-17 (δ$_H$ 1.75) and H-12 (δ$_H$ 5.65) in association with the chemical shifts of C-16 (<20 ppm) and C-17 (>20 ppm) (Lange & Lee 1986). Accordingly, the structure of 1 was determined as sibogin C.

1D NMR spectra of 2 were closely similar to 1. The major difference was the lack of an olefinic double bond between C-6 and C-7 (δ$_H$ 5.45; δ$_C$ 129.9, 126.6) in 1, and the corresponding addition of an oxygenated methine (δ$_H$ 4.52; δ$_C$ 74.9) and an oxygenated quaternary carbon (δ$_C$ 75.6) groups in 2. Its HR-ESI-MS data (m/z 433.2567 [M + Na]$^+$) provided the molecular formula of C$_{23}$H$_{38}$O$_6$, more two OH units than 1, indicating that the additional oxygenated substitutions in 2 could be in the form of OH groups. The HMBC correlations from H$_3$-16 (δ$_H$ 1.16) to C-6 (δ$_C$ 74.9) and C-7 (δ$_C$ 75.6) further confirmed the oxygenation occurring at C-6 and C-7 positions, in adjacent diols. Similar NOE relationships indicated that 2 possesses the same relative configurations as 1 except for the 6,7 diols. The trans-configuration of 6,7-diols in 2 were deduced from the key NOE correlation from H-6 (δ$_H$ 4.52) to H$_3$-15 (δ$_H$ 1.65) and H$_3$-8 (δ$_H$ 1.71), and from H$_3$-16 (δ$_H$ 1.16) to H$_3$-8 (δ$_H$ 1.90), together with the similar coupling constant of H-6 (δ$_H$ 4.52, br d, $J = 12.2$ Hz) and optical rotation value ([α]$_D^{20} = -43.6$) as co-isolated 7 (Lai et al. 2012) and 8 (Zhou et al. 2014). Thus, the structure of 2 was ascertained as the oxygenated product of 1 at C-6 and C-7 and named as sibogin D.

The known compounds were identified as ophirin (3) (Kashman 1980), 13-deacetyloxy-caliciphorin B (4) (Seo et al. 1997), caliciphorin B (5) (Ochi et al. 1991), muricellin (6) (Seo et al. 2000), astrogargin H (7) (Lai et al. 2012), sibogin A (8) (Zhou et al. 2014), astrogargin D (9) (Lai et al. 2012) and astrogargin E (10) (Lai et al. 2012), by comparison of their spectral data with the reported values.

All the isolates (1–10) were tested for cytotoxic activities against four selected tumour cell lines (HCT116, P388, HL-60 and K562 cell lines). Compound 4 showed a weak cytotoxicity with an IC$_{50}$ value of 29.1 µM against HL-60 cell line, and the other compounds showed inactive of cytotoxicity against the selected tumour cell lines. Interestingly, compounds 3 and 5 showed significant antifouling activity against the green mussel P. viridis, a representative marine fouling organism, with EC$_{50}$ values of 1.8 and 18.1 µg/mL, and LC$_{50}$ values of 33.4 and >50 µg/mL, respectively. Also, it was noticed that the tested EC$_{50}$ values of both 3 and 5 were lower than the criterion of 25 µg/mL (Rittschof et al. 2003) and possessed a relatively large therapeutic ratio (LC$_{50}$/EC$_{50}$ = 18.6 for 3, >27.6 for 5), much larger than the positive control (Cu$^{2+}$, EC$_{50}$ and LC$_{50}$ = 1.0 µg/mL, LC$_{50}$/EC$_{50}$ = 1.0), indicating that 3 and 5 could be considered as potent antifouling agents against the green mussel P. viridis.

3. Experimental
3.1. General

Optical rotations were measured on a JASCO P-1020 digital polarimeter (JASCO Corporation, Tokyo, Japan). IR spectra were recorded on a Nicolet Nexus 470 (FT-IR) spectrophotometer
(Nicolet, Madison, WI, USA), KBr pellets. NMR spectra were recorded on a JEOL JNMECP 600 instrument, 600 MHz for $^1$H NMR and 150 MHz for $^{13}$C NMR in CDCl$_3$, chemical shifts $\delta$ in ppm referred to the solvent peaks at $\delta$H 7.26 and $\delta$C 77.0 for CDCl$_3$ and coupling constant $J$ in Hz (JEOL Ltd, Tokyo, Japan). HR-ESI-MS and LC–MS were obtained from a Micromass Q-Tof Ultima GLOBAL GAA076 LC-mass spectrometer (Waters Corporation, Milford, MA, USA). HPLC separation was performed on an Agilent 1100 series instrument with DAD detector (Agilent Technologies, Palo Alto, CA, USA), equipped with a semi-preparative reversed-phased columns (YMC-packed C18, 5 $\mu$m, 250 mm $\times$ 10 mm, 1.5 mL/min). Precoated silica gel plates (GF$_{254}$, Qingdao Marine Chemical Inc., Qingdao, P.R. China) were used for TLC analyses. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, P.R. China) was used for column chromatography (CC).

3.2. Animal material

The gorgonian $M$. sibogae (Nutting) featured with dark red colour was collected from the coral reef regions of Weizhou Island (21°3’0”N, 109°7’0”E), at a depth ranging from 18 to 25 m, Guangxi Province, P.R. China, in November 2010. The specimen was identified by Prof. Ren-Lin Zou (the South China Sea Institute of Oceanology, Chinese Academy of Sciences). A voucher of the specimen (No. WZD2010-3) was deposited in the School of Medicine and Pharmacy, Ocean University of China, P.R. China.

3.3. Extraction and isolation

The frozen specimen (5.0 kg, wet weight; 3.8 kg, the residue after extraction) was homogenised and extracted with MeOH for three times at room temperature (each, 5 L, 3 d). The combined extraction solution was evaporated in reduced pressure and desalinated for three times to yield a residue (87.0 g). The residue was then subjected to a silica gel vacuum CC eluted with acetone–petroleum ether (from 0:1 to 1:0 gradient), to give 10 fractions. Fractions 2 and 3 were found to contain eunicellin-based diterpenoid constituents by careful analysis. The two fractions were further separated by repeated silica gel CC, guided by characteristic TLC colour for eunicellin-based diterpenoids (bright-red or orange), followed by semi-preparative HPLC (reverse phase C$_{18}$, MeOH/H$_2$O, 67/33 and 70/30) to provide 1 (23.0 mg), 2 (1.9 mg), 3 (9.0 mg), 4 (5.4 mg), 5 (3.2 mg), 6 (4.1 mg), 7 (4.7 mg), 8 (5.2 mg), 9 (5.1 mg) and 10 (4.5 mg).

3.3.1. Sibogin C (1)

C$_{23}$H$_{36}$O$_4$, colourless oil. [$\alpha$]$_D^{20}$ = -52.8 (c = 0.1, MeOH). IR (KBr) $\nu$max: 2964, 2817, 2356, 1733, 1650, 1458, 1368, 1245, 1078, 1022 cm$^{-1}$. HR-ESI-MS $m/z$: 399.2504, [M + Na]$^+$ (calcd for C$_{23}$H$_{36}$O$_4$Na$^+$, 399.2506).

$^1$H NMR (CDCl$_3$, 600 MHz): $\delta$ 5.65 (1H, d, $J$ = 4.8 Hz, H-12), 5.45 (1H, dd, $J$ = 11.0, 6.4 Hz, H-6), 4.42 (1H, d, $J$ = 6.6 Hz, H-2), 4.20 (1H, dd, $J$ = 5.0, 4.2 Hz, H-9), 3.59 (1H, br s, H-13), 3.29 (3H, s, OMe), 2.49 (1H, dd, $J$ = 13.6, 5.9 Hz, H$_a$-8), 2.44 (1H, m, H-1), 2.40 (1H, br d, $J$ = 8.5 Hz, H-10), 2.36 (1H, m, H$_b$-5), 2.27 (1H, ddd, $J$ = 13.5, 5.5, 3.1 Hz, H$_b$-4), 2.13 (1H, m, H$_b$-5), 2.03 (1H, br d, $J$ = 13.6 Hz, H$_b$-8), 2.01 (1H, m, H$_b$-4), 1.95 (3H, s, OAc), 1.92 (1H, m, H-14), 1.79 (3H, s, H-16), 1.75 (3H, s, H-17), 1.72 (3H, s, H-15), 1.61 (1H, m, H-18), 0.99 (3H, d, $J$ = 6.6 Hz, H-19), 0.91 (3H, d, $J$ = 6.6 Hz, H-20).

$^{13}$C NMR (CDCl$_3$, 150 MHz): $\delta$ 169.6 (C, OAc), 137.1 (C, C-11), 129.8 (CH, C-6), 126.1 (C, C-7), 121.9 (CH, C-12), 89.6 (C, C-3), 88.5 (CH, C-2), 81.8 (CH, C-9), 76.1 (CH, C-13), 55.5 (CH$_3$, OMe), 47.0 (CH, C-10), 44.2 (CH$_2$, C-8), 41.3 (CH, C-14), 39.8 (CH, C-10), 31.8 (CH$_2$, C-4), 30.5 (CH, C-18), 23.0 (CH$_3$ OAc), 22.8 (CH$_2$, H-5), 22.3 (CH$_3$, C-17), 21.9 (each CH$_3$, C-19, C-15), 20.5 (CH$_3$, C-20), 19.0 (CH$_3$, C-16).
3.3.2. Sibogin D (2)

C_{23}H_{38}O_{6}, colourless oil. \([\alpha]_D^{20} = -43.6 (c = 0.1, \text{MeOH})\). IR (KBr) \(\nu_{\text{max}}\): 3443, 2962, 2925, 2854, 1734, 1682, 1649, 1508, 1458, 1245, 1076, 1026 cm\(^{-1}\). HR-ESI-MS \(m/z\): 433.2567, [M + Na]\(^+\) (calcd for C_{23}H_{38}O_{6}Na\(^+\), 433.2561).

\(^1\)H NMR (CDCl\(_3\), 600 MHz): \(\delta\) 5.71 (1H, d, \(J = 4.5\) Hz, H-12), 4.52 (each 1H, dd, \(J = 12.0, 3.1\) Hz, H-6, H-9), 4.44 (1H, d, \(J = 7.0\) Hz, H-2), 3.64 (1H, d, \(J = 4.5\) Hz, H-13), 3.30 (3H, s, OMe), 2.70 (1H, td, \(J = 7.2, 2.6\) Hz, H-1), 2.31 (1H, dt, \(J = 14.8, 4.9\) Hz, H\(_a\)-4), 2.25 (1H, br d, \(J = 7.8\) Hz, H-10), 2.09 (1H, td, \(J = 14.8, 4.6\) Hz, H\(_b\)-4), 2.01 (3H, s, OAc), 1.99 (1H, br s, H-14), 1.90 (each 1H, m, H\(_a\)-8, H\(_a\)-5), 1.76 (3H, s, H-17), 1.71 (1H, br d, \(J = 15.0\) Hz, H-8), 1.65 (3H, s, H-15), 1.51 (1H, m, H-18, H\(_b\)-5), 1.16 (3H, s, H-16), 1.01 (3H, d, \(J = 7.1\) Hz, H-19), 0.95 (3H, d, \(J = 6.6\) Hz, H-20). \(^{13}\)C NMR (CDCl\(_3\), 150 MHz): \(\delta\) 169.7 (C, OAc), 136.8 (C, C-11), 122.9 (CH, C-12), 86.7 (C, C-3), 85.7 (CH, C-2), 77.5 (CH, C-9), 76.0 (CH, C-13), 75.6 (C, C-7), 74.9 (C, C-6), 55.5 (CH\(_3\), OMe), 48.1 (CH, C-10), 46.8 (CH\(_2\), C-8), 41.6 (CH, C-14), 39.2 (CH, C-1), 30.4 (CH, C-18), 29.7 (CH\(_2\), C-4), 29.1 (CH\(_2\), C-5), 23.8 (CH\(_3\), C-15), 22.6 (CH\(_3\), C-20), 22.0 (each CH\(_3\), C-16, OAc), 21.9 (CH\(_3\), C-17), 21.0 (CH\(_3\), C-19).

3.4. Bioactivity testing

3.4.1. Cytotoxicity testing

Cytotoxicities against HL-60 (human promyelocytic leukemia), P388 (murine leukemia), K562 (human leukemia) and HCT116 (human colorectal cancer) cell lines were tested by MTT (Alley et al. 1988) and SRB (Skehan et al. 1990) methods, respectively, using Adriamycin (doxorubicin, ADM) as a positive control.

3.4.2. Antifouling activity testing

Antifouling test against the green mussel *P. viridis* was carried out according to literature procedures (Feng et al. 2013). Briefly, the green mussels with shell length of 15–25 mm were chosen for the test, with particular care taken to cut the byssal mass (stem and old threads) off each mussel with sharp scissors. The compounds were dissolved in dimethylsulfoxide (DMSO). One mussel was placed in each well (24 well plates) containing 20 \(\mu\)L of each compound solution and 2 mL filtered (0.22 \(\mu\)m) seawater (FSW). Ten replicates were set up for each of the treatment groups and the negative control (20 \(\mu\)L DMSO added to 2 \(\mu\)L FSW). Cu\(^{2+}\) was used as a positive control. After 24 h of incubation, the number of byssus threads produced by each mussel and the number of dead mussels were counted.

4. Conclusions

A re-study on *M. sibogae* targeted at the eunicellin-based diterpenoids resulted in the isolation of two new eunicellin-based diterpenoids, sibogins C (1) and D (2), and eight known analogues. The new findings enriched the eunicellin-based diterpenoid structural diversity and provided more evidences for chemical marks of the genus *Muricella*. In addition, compounds 3 and 5 were proved to possess potent antifouling activity against the green mussel *P. viridis*. This was the first time to report the antifouling activity of eunicellin-type diterpenoids against green mussel, extending the antifouling spectrum of the class of compounds.

Supplementary material

Supplementary material relating to this article is available online, including 2D NMR correlations for compound 1, NMR and MS spectra for 1 and 2, alongside Figures S1–S15.
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Disclosure statement
No potential conflict of interest was reported by the authors.

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Note
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