Cytotoxic Polyacetylenes from the Red Sea Sponge *Siphonochalina siphonella*

Seif-Eldin N. Ayyad\(a,b,\ast\), Rihab Angawy\(a\), Walied M. Alarif\(c,d\), Esraa A. Saqer\(a\), and Farid A. Badria\(e\)

\(a\) Department of Chemistry, Faculty of Science, King Abdulaziz University, P. O. Box 80203, Jeddah 21589, Saudi Arabia. Fax: +966 2 6951599. E-mail: snayyad2@yahoo.com

\(b\) Permanent address: Department of Chemistry, Faculty of Science, Damietta University, Damietta, Egypt

\(c\) Department of Marine Chemistry, Faculty of Marine Sciences, King Abdulaziz University, P. O. Box 80207, Jeddah 21589, Saudi Arabia

\(d\) Forensic Chemistry Laboratory at Mansoura, Ministry of Justice, Mansoura, Egypt

\(e\) Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

\(\ast\) Author for correspondence and reprint requests


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Two new polyacetylenes, callyspongenol-D (1) and callyspongendiol (2), the known polyacetylene dehydrosiphonochalynol (3), and the known triterpenoid sipholenol-A (4) were isolated from the Red Sea sponge *Siphonochalina siphonella*. Their chemical structures were elucidated on the basis of spectroscopic analyses. The cytotoxicity of the isolated compounds towards the human mammary carcinoma cell line MCF-7 was determined by the lactate dehydrogenase (LDH) assay, and compounds 4 and 1 were found to be the most toxic of the four, with IC\(_{50}\) values of 8.8 and 11.7 µM, respectively.

**Key words:** Red Sea, Marine Sponges, Breast Cancer

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**Introduction**

Breast cancer is the fifth most common cause of cancer-related deaths worldwide (Ferlay *et al*., 2010). In India, it is the second most common cancer among women. Accordingly, there is a need for the development of novel anticancer drugs, drug combinations, and treatment methods to effectively treat these patients (Sandhya and Mishra, 2006; Fisher *et al*., 1998; Gajalakshmi *et al*., 1997). The etiology of such diseases is multifactorial; hormonal, genetic, and environmental factors appear to interact in the pathogenesis of breast cancer (Russo *et al*., 2000). It has been proven that a number of commercial food additives, pesticides, and industrial chemicals are carcinogenic in both animals and humans (IARC, 1971 – 1985). Prevention could reduce the breast cancer risk and potentially reduce the number of breast cancer-related deaths. Current treatments used in breast cancer therapy, such as radiation, antihormonal therapy, surgery, and chemotherapy using synthetic drugs, have been reported to produce various side effects. Considering these facts, attention has focused on the identification of naturally occurring compounds as possible chemopreventive agents (Wattenberg, 1985; Huang *et al*., 1983). Hence, the search for novel drugs of natural origin, including the marine environment, is desirable. Recently, our group reported a number of acetylenic compounds isolated from the Red Sea sponge *Haliclona* sp. (Alarif *et al*., 2013) and their activities against the breast cancer cell line MCF-7. In continuation of this previous work, we investigated the constituents of the marine sponge *Siphonochalina siphonella*. These efforts resulted in the isolation of four metabolites, of which compounds 1 and 2 are new polyacetylenes, compound 3 is a known polyacetylene, and lastly compound 4 is the known triterpenoid sipholenol-A.
Results and Discussion

The diethyl ether-soluble fraction derived from the crude extract of dried sponge material was fractionated and purified by conventional chromatographic techniques to give three acetylenes, 1–3 (Fig. 1), in addition to the triterpenoid compound 4.

Callyspongenol-D (1) was obtained as a pale yellow oil with the molecular formula C_{20}H_{24}O as established by HRFABMS. Combination of $^{13}$C NMR spectrometry.
try with an HSQC experiment revealed the presence of eight acetylenic carbon atoms in 1, including seven singlets and one doublet, two protonated olefinic carbon atoms, an oxygenated methylene of a terminal carbinol carbon atom, along with signals for nine paraffinic methylene carbon atoms (Table I).

Based on the above data, compound 1 contains nine degrees of unsaturation; eight of them accounted for four acetylenic groups, and the ninth is for a carbon-carbon double bond. Hence, 1 should be acyclic. The $^1$H NMR spectrum featured the following: a terminal acetylenic proton signal appeared as doublet with a J value of 1.8 Hz from a long-range effect resonating at $\delta_H$ 3.11 ppm (H-20), a terminal primary alcohol proton signal as double triplet with J values of 6.0 and 1.8 Hz, originating from a geminal effect and a long-range effect of C-4, respectively ($\delta_H$ 4.29 ppm, H-2), and a cis-disubstituted double bond, originating from two correlated olefinic protons (J = 10.8 Hz), showed two signals, the first as double triplet and the second as double doublet at $\delta_H$ 6.01 and 5.50 ppm, respectively. The COESY and HSQC experiments allowed the assignment of the subunits C-1/C-8 and C-13/C-20 within 1, which were substantiated by HMBC data. HMBC correlations of H$_2$-8/C-9, H$_2$-8/C-10, H$_2$-13/C-12, and H$_2$-13/C-11 established the unambiguous placement of the conjugated diyne moiety at C-9/C-12. The structure elucidation of 1 based on NMR data was further confirmed by mass spectral analysis. EIMS data of 1 exhibited several conspicuous fragments containing linear alkyl chains (Youssef et al., 2003). Fragments observed at m/z 125, 155, 107, 93, and 65 supported the structure assigned for 1 (Fig. 2). Accordingly, compound 1 was assigned the structure (17Z)-1-hydroxyicos-17-ene-2,9,11,19-tetrayne (callyspongenol-D).

Callyspongenediol (2) was obtained as a yellow oil with the molecular formula C$_{30}$H$_{44}$O$_2$ as established by HRFABMS. Its $^{13}$C NMR spectrum, together with DEPT and HSQC experiments, revealed the presence

Table I. $^1$H (CDCl$_3$, 600 MHz) and $^{13}$C (CDCl$_3$, 150 MHz) NMR spectral data of 1 and 2.

<table>
<thead>
<tr>
<th>No.</th>
<th>$\Delta_H$ (J in Hz)</th>
<th>$\Delta_C$</th>
<th>$\Delta_H$ (J in Hz)</th>
<th>$\Delta_C$</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4.29 (dt, 6.0, 1.8)</td>
<td>51.6 (t)</td>
<td>2.57 (d, 1.8)</td>
<td>74.2 (d)</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>78.8 (s)</td>
<td>–</td>
<td>83.6 (s)</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>86.3 (s)</td>
<td>4.84 (brd, 5.4)</td>
<td>63.1 (d)</td>
</tr>
<tr>
<td>4</td>
<td>2.26 (tt, 6.6, 1.8)</td>
<td>18.7 (t)</td>
<td>5.61 (dd, 15.0, 6.0)</td>
<td>128.6 (d)</td>
</tr>
<tr>
<td>5</td>
<td>1.25 (m)</td>
<td>27.8 (t)</td>
<td>5.92 (dt, 15.0, 6.0)</td>
<td>134.8 (d)</td>
</tr>
<tr>
<td>6</td>
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<td>27.7 (t)</td>
<td>2.07 (m)</td>
<td>32.1 (t)</td>
</tr>
<tr>
<td>7</td>
<td>1.25 (m)</td>
<td>27.5 (t)</td>
<td>1.37 (m)</td>
<td>29.3 (t)</td>
</tr>
<tr>
<td>8</td>
<td>2.33 (t, 7.2)</td>
<td>19.3 (t)</td>
<td>1.25 (m)</td>
<td>29.7 (t)</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>79.2 (s)</td>
<td>1.25 (m)</td>
<td>29.7 (t)</td>
</tr>
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<td>10</td>
<td>–</td>
<td>66.1 (s)</td>
<td>1.66 (p, 6.6)</td>
<td>29.2 (t)</td>
</tr>
<tr>
<td>11</td>
<td>–</td>
<td>66.0 (s)</td>
<td>2.25 (tt, 7.2, 2.4)</td>
<td>18.2 (t)</td>
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<td>–</td>
<td>79.2 (s)</td>
<td>–</td>
<td>81.0 (s)</td>
</tr>
<tr>
<td>13</td>
<td>2.35 (t, 7.2)</td>
<td>19.4 (t)</td>
<td>–</td>
<td>79.5 (s)</td>
</tr>
<tr>
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<td>28.1 (t)</td>
<td>2.13 (tt, 7.2, 2.4)</td>
<td>19.0 (t)</td>
</tr>
<tr>
<td>15</td>
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<td>1.47 (p, 7.2)</td>
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</tr>
<tr>
<td>16</td>
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<tr>
<td>17</td>
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<td>1.65 (p, 6.6)</td>
<td>29.2 (t)</td>
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<td>29.7 (t)</td>
<td>29.7 (t)</td>
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<td>29.7 (t)</td>
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</tr>
<tr>
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<td>29.7 (t)</td>
<td>29.7 (t)</td>
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<tr>
<td>25</td>
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<td>1.25 (m)</td>
<td>29.7 (t)</td>
<td>29.7 (t)</td>
</tr>
<tr>
<td>26</td>
<td>–</td>
<td>1.47 (p, 7.2)</td>
<td>–</td>
<td>29.6 (t)</td>
</tr>
<tr>
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<td>1.71 (q, 7.2)</td>
<td>–</td>
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<tr>
<td>28</td>
<td>4.37 (dt, 6.6, 1.8)</td>
<td>–</td>
<td>62.6 (d)</td>
<td>85.2 (s)</td>
</tr>
<tr>
<td>29</td>
<td>–</td>
<td>–</td>
<td>62.6 (d)</td>
<td>85.2 (s)</td>
</tr>
<tr>
<td>30</td>
<td>–</td>
<td>2.46 (d, 1.8)</td>
<td>73.1 (d)</td>
<td>–</td>
</tr>
</tbody>
</table>
of eight acetylenic carbon atoms including six singlets and two doublets, two protonated olefinic carbon atoms, two oxygenated methine carbon atoms for two secondary alcohol groups, and signals for eighteen methylene carbon atoms (Table 1).

From the previous discussion, four acetylenic groups and one double bond accommodated all degrees of unsaturation, therefore 2 is acyclic. The $^1$H NMR spectrum displayed: two doublet signals for two terminal acetylenic protons at $\delta_H$ 2.57 ppm (d, $J = 1.8$ Hz, H-1) and $\delta_H$ 2.46 ppm (d, $J = 1.8$ Hz, H-30); two oxygenated methine proton signals, including a doublet resonating at $\delta_H$ 4.84 ppm (H-3) with a $J$ value of 5.4 Hz, originating from a vicinal effect, and the second appeared as double triplet signal resonating at $\delta_H$ 4.37 ppm (H-28) with $J$ values of 6.6 and 1.8 Hz, originating from both a vicinal and a long-range effect, respectively, of H-30; and two olefinic proton signals, one appearing as a double doublet and the other one as a double triplet resonating at $\delta_H$ 5.61 and 5.92 ppm, respectively. The $E$-geometry of the olefinic moiety in 2 was concluded from the $^1$H-$^1$H coupling constant of $J = 15.0$ Hz. HMBC correlations of H$_2$-14/C-13, H$_2$-14/C-12, H$_2$-16/C-17, and H$_2$-16/C-18, along with the peaks observed in the EI mass spectrum at $m/z$ 255 and 165 were indicative of the subunit C-12/C-18 and also established the unambiguous placement of the non-conjugated diyne moiety at C-12/C-17. Moreover, the subunit C-1/C-6 was established from the COESY and HMBC data, where correlations of H-3/C-1 and C-5, H-5/C-6 and C-3, H-3/H-4 and H-5/H-6 were noticed. Again, the subunit C-1/C-6 can be extended to C-1/C-11 with the aid of EIMS, where fragments at $m/z$ 165, 137, 109, and 95 were analysed (Fig. 2). Finally, the correlations of H-28/C-30 and C-27 observed in the HMBC spectrum and H-30/H-28/H-27 in COESY along with fragments observed at $m/z$ 181 and consecutive CH$_2$ losses established the subunit C-19/C-30. Accordingly, compound 2 was assigned the

Fig. 2. Prominent mass spectral fragment peaks ($m/z$) of 1 and 2.
structure (4E)-3,28-dihydroxytriconta-4-ene-1,12,17, 29-tetrayne (callyspongendiol).

Compounds 3 and 4 were identified as dehydro-
siphonochalynol and sipholenol-A, respectively, by
comparison of their spectral data with those in the lit-
erature (Rotem and Kashman, 1979; Carmely et al.,
1983).

Two main pathways leading to cell death have been
identified in normal and disease conditions, i.e. apo-
ptosis and necrosis. Several assays are known for the
evaluation of apoptosis, while relatively few assays are
available for measuring necrosis. A specific feature of
necrotic cells is the permeabilization of the plasma
membrane, and the extent of the permeabilization can
be estimated in tissue culture settings by measuring the
level of released intracellular enzyme lactate dehydro-
genase (LDH) (Chan et al., 2013; Uchide et al., 2009).
Extracellular LDH is a reliable marker for cytotoxic-
ity because damaged cells are fragmented completely
during the course of prolonged incubation and thereby
release LDH. In addition, the LDH assay is used to as-
ssess plasma membrane integrity. Generally, in cancer
cells the release of LDH is absent due to the propaga-
tion of the cells (Sivalokananthan et al., 2006).

Cells of the MCF-7 line were incubated for 1, 24,
48, and 72 h in medium containing the isolated com-
ounds at different concentrations, and their growth
was compared with that of untreated cells and of cells
treated with three well known cytotoxic compounds
[5-fluorouracil (5-FU), methotrexate, cisplatin] com-
monly used in the chemotherapy of breast cancer (Ta-
ble II). Compound 4 exhibited the highest cytotoxic ac-
tivity (IC$_{50}$ 8.8 µM) against the breast cancer cell line
MCF-7.

Metabolite 1 was second in cytotoxicity as deter-
dined by the LDH activity (11.7 µM). Finally, com-
ounds 2 and 3 showed the lowest cytotoxicity at 65.7
and 73.6 µM, respectively, at all periods.

The term acetylenic natural products describes
a class of compounds with alkynyl functionality, which
are widely distributed among marine and terrestrial or-
ganisms. These compounds are derived from polyke-
tide and fatty acid precursors (Minto and Blacklock,
2008) and possess a wide spectrum of biochemical and
ecological properties. Polyacetylenic compounds can
be cytotoxic via inhibition of mitochondrial respiration
(Matsunaga et al., 1990, 1995). While other evidence
suggested that acetylenes can function as sensitizers
for the production of singlet oxygen in photosystem I,
most polyacetylenes have light-independent functions
(Hansen and Boll, 1986). An example of a bioactive
polyacetylenic alcohol is panaxytriol (isolated from
Panax ginseng) that potentiates the cytotoxicity of mit-
omycin C in chemotherapy (Matsunaga et al., 1994).
Moreover, oenanthotoxin is a bioactive polyacetylene
that targets the central nervous system and has been
recognized since the 12th century as a useful poison
for fish and other animals. Only few studies have ad-
dressed the biological functions and the mechanism(s)
of action of acetylenic natural products, individually or
as a group. The majority of reports on polyacetylene
bioactivities relate to activities in assays for antibiotic
and anticancer agents.

**Materials and Methods**

**General**

Optical rotations were measured on a ATAGO
POLAX-L 2 polarimeter (Rudolph Research Analytical,
Hackettstown, NJ, USA). EIMS analyses were car-
ried out on a Shimadzu-QP 2010 instrument (Ky-
oto, Japan). 1D and 2D NMR spectra were recorded in
CDCl$_3$ on a Bruker AVANCE III WM 600 MHz spec-
trometer (Karlsruhe, Germany); $^{13}$C NMR spectra at
150 MHz. Chemical shifts are given in $\delta$ (ppm) relative
to tetramethylsilane (TMS) as internal standard. Thin-
layer chromatography (TLC) was performed on silica
gel GF 254 (Merck, Darmstadt, Germany) of 0.25 mm
layer thickness. The sponge Siphonochalina siphonella
was collected from Sharm Obhur, Jeddah, Saudi Ara-
bia, and was identified by Dr. Yahia Folos (Faculty of
Marine Sciences, King Abdulaziz University, Jed-
dah, Saudi Arabia). A voucher sample (JAD 04070)
has been deposited at the Marine Chemistry Depart-
ment, King Abdulaziz University, Jeddah, Saudi Ara-
bia.

**Extraction and isolation of compounds**

The dried sponge material (63.7 g) was extracted
with 2 × 6 L of CH$_2$Cl$_2$/MeOH (1:1, v/v), 24 h

<p>| Table II. IC$_{50}$ values of compounds 1–4 on MCF-7 by the LDH assay, after 72 h. |</p>
<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ [µM]$^a$</th>
<th>Positive control</th>
<th>IC$_{50}$ [µM]$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.7 ± 0.39</td>
<td>5-FU</td>
<td>2.83 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>65.7 ± 1.06</td>
<td>Methotrexate</td>
<td>0.089 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>73.6 ± 0.48</td>
<td>Cisplatin</td>
<td>62 ± 0.049</td>
</tr>
<tr>
<td>4</td>
<td>8.8 ± 0.79</td>
<td></td>
<td>8.0 ± 0.29</td>
</tr>
</tbody>
</table>

$^a$ Data shown are the mean ± SD of three experiments. The means
were significantly different across the samples.
for each batch, at room temperature. The extract was concentrated under reduced pressure to provide 18.75 g of a viscous oil. The residue was partitioned between diethyl ether and water, the organic layer was homogenized with small amounts of aluminum oxide and CHCl₃, poured onto an aluminum oxide column (diameter, 50 cm; length, 100 cm) which was eluted with gradients from n-hexane to diethyl ether and from n-hexane to ethyl-acetate; 150 fractions (∼ 25 mL each) were collected. Similar fractions were pooled, according to their TLC behaviour, into eight samples (P-A to P-H), employing a UV₂₅₄ lamp and/or 50% sulfuric acid in ethanol as spraying reagent for detection of spots. All compounds were purified by preparative TLC (PTLC) and re-purified by employing Sephadex LH-20 (Amersham Pharmacia, Uppsala, Sweden) – if appropriate – using a mixture of MeOH/CHCl₃ (9:1).

Sample P-A (n-hexane/diethyl ether, 6:4; 22 mg) was purified by PTLC on aluminium oxide using n-hexane/diethyl ether (7:3), yielding 3 (15 mg). Sample P-B (n-hexane/diethyl ether, 6:4; 25 mg) was purified by PTLC on aluminium oxide using n-hexane/diethyl ether (7:3) yielding 1 (23 mg). Sample P-C (n-hexane/diethyl ether, 4:6; 15 mg) was purified by PTLC on aluminium oxide using n-hexane/diethyl ether (1:1), yielding two compounds, i.e. 2 (Rf 0.45; 3 mg) and 4 (Rf 0.31; 5 mg).

Callyspongenol-D (1): Yellow oil. – Yield: 23 mg (0.036%, dry weight). – Rf = 0.28 [n-hexane/diethyl ether (7:3), brown colour with sulfuric acid/methanol]. – IR (film): νmax = 3361.11, 3292.19, 2936.22, 2860.42, 2825.46, 2215.5. 1615.91, 1456.05, 1424.17, 1357.24, 1258.49, 1006.87, 895.92, 739.16 cm⁻¹. – Positive HRFABMS: m/z = 303.1733 [M+Na]+ (calcd. for C₁₂H₂₃ONa, 303.1725). – EIMS: m/z = 280, 225, 211, 197, 183, 169, 155, 125, 107, 93, 79, 65. – ¹H and ¹³C NMR: see Table I.

Callyspongiadiol (2): Straw-yellow oil. – Yield: 5 mg (0.0078%, dry weight). – Rf = 0.31 [n-hexane/ethyl acetate (6:4), violet appearance under UV light, brown spot developing upon spraying with 50% sulfuric acid in ethanol]. – [α]D = +2.1° (CH₂Cl₂; c = 0.01). – IR (film): νmax = 3365.07, 3292.39, 2924.90, 2851.77, 2113.17, 1668.86, 1463.93, 1432.44, 1312.99, 1261.39, 1009.1, 967.53, 904.30 cm⁻¹. – Positive HRFABMS: m/z = 459.3227 [M+Na]+ (calcd. for C₂₃H₄₄O₂Na, 459.3229). – EIMS: m/z = 436, 381, 367, 353, 325, 297, 283, 271, 181, 165, 137, 109, 95, 54, 28. – ¹H and ¹³C NMR: see Table I.

Dehydrospishonochalynol (3): It was identified by comparison of its spectral data with those in the literature (Rotem and Kashman, 1979).

Sipholenol-A (4): It was identified by comparison of its spectral data with those in the literature (Carmely et al., 1983).

Cell line and culture conditions

The non-aggressive human mammary epithelial breast cancer cell line MCF-7 was purchased from VACSERA (Cairo, Egypt) and cultured in RPMI medium (Roswell Park Memorial Institute, Buffalo, NY, USA) supplemented with 100 µg/mL penicillin-streptomycin, 2.5 µg/mL fungizone, 10% heat-activated foetal calf serum, and 2 mM glutamine. Cells were allowed to grow at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air to form a monolayer. At 60–70% confluence, cells were subcultured; first they were washed with phosphate-buffered saline (PBS), then trypsinized with 3 mL of 0.25% trypsin in 0.03% EDTA, then washed with fresh medium and seeded at 1·10⁴ cells/well in a 96-well microplate.

The reagent kit for the assay of LDH (lactate dehydrogenase) was purchased from Biorex Diagnostics (Antrim, UK). Methotrexate, 5-FU, and cisplatin were kindly supplied as a gift from the Oncology Center, Mansoura University, Mansoura, Egypt.

Cell treatment

MCF-7 cells were treated with the isolated compounds at concentrations from 0–250 μM. All chemicals were dissolved in RPMI medium and filtered through a membrane filter (0.2 µm) before cell treatment. LDH activities were measured at 1, 24, 48, and 72 h.

Cytotoxicity assay

Cytotoxicity was evaluated through monitoring the release of LDH into the medium. Fifty µL of the supernatant were drawn off from each cell culture well and assayed for LDH activity by measuring the absorbance at 340 nm (Hilf et al., 1976).

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