Regular Article

**Anthraquinone and Butenolide Constituents from the Crinoid *Capillaster multiradiatus***

Le Thi Vien\textsuperscript{a,b}, Tran Thi Hong Hanh\textsuperscript{a}, Phan Thi Thanh Huong\textsuperscript{a}, Nguyen Hai Dang\textsuperscript{a},
Nguyen Van Thanh\textsuperscript{a}, Nguyen Xuan Cuong\textsuperscript{a,h,*}, Nguyen Hoai Nam\textsuperscript{a},
Do Cong Thung\textsuperscript{c}, Phan Van Kiem\textsuperscript{a}, and Chau Van Minh\textsuperscript{a,*}

\textsuperscript{a} Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST),
18 Hoang Quoc Viet, Caugiay, Hanoi 100000, Vietnam

\textsuperscript{b} Graduate University of Science and Technology, VAST, 18 Hoang Quoc Viet, Caugiay, Hanoi 100000, Vietnam

\textsuperscript{c} Institute of Marine Environment and Resources (IMER), VAST, 246 Da Nang, Haiphong 180000, Vietnam

* To whom correspondence should be addressed:

E-mail: cvminh@vast.vn (Minh C. V.)

E-mail: cuongnx@imbc.vast.vn (Cuong N. X.)
Abstract

Seven anthraquinones including two new compounds namely capillasterquinones A and B (1 and 2) and one new butenolide namely capillasterolide (8) were isolated and structurally elucidated from the crinoid *Capillaster multiradiatus*. The inhibitory effect of compounds 1–8 on lipopolysaccharide (LPS)-induced nitric oxide (NO) production as well as inhibition of 1 on expressions of iNOS and COX-2 proteins in RAW264.7 cells were also evaluated. As the obtained results, capillasterquinone A (1) showed strong NO production inhibitory activity with an IC$_{50}$ of 5.89 ± 0.11 µM. In addition, compound 1 reduced the LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expressions in a dose-dependent manner.

**Keywords:** *Capillaster multiradiatus*; crinoid; anthraquinone; NO production; iNOS; COX-2
The phylum Echinodermata is divided into five classes: Asteroidea (starfish or sea stars), Ophiuroidea (brittle stars), Crinoidea (feather stars and sea lilies), Echinoidea (sea urchins), and Holothuroidea (sea cucumbers). The chemotaxonomic relations observed in this phylum are particularly clear; each class is characterized by a particular set of secondary metabolites that is probably specific to the class. For the class Crinoidea, these are anthraquinonic pigments which have been found in all species studied. Among crinoids, *Capillator* is a little investigated genus with two known naphthopyrones TMC-256A1 and 5,8-dihydroxy-6-methoxy-2-propyl-4H-naphtho[2,3-b]pyran-4-one reported from the species *Capillator multiradiatus*. Recently, the acetone, butanol, chloroform, ethyl acetate, and methanol extracts of this species were found to exhibit antibacterial activity against several human and fish bacterial pathogens.

As part of our ongoing investigations on chemical constituents and biological activities of the Vietnamese echinoderms, this paper deals with the isolation and structure elucidation of seven anthraquinones including two new compounds namely capillasterquinones A and B (1 and 2) and one new butenoide namely capillasterolide (8) from the crinoid *C. multiradiatus*. The inhibitory effect of 1–8 on lipopolysaccharide (LPS)-induced nitric oxide (NO) production as well as inhibition of 1 on expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) proteins in RAW264.7 cells were also evaluated.

**Result and Discussion**

Using various chromatographic separations, seven anthraquinones and one butenoide (1–8, Fig. 1) were isolated from a methanol extract of the crinoid *C. multiradiatus*. Detailed analysis of the spectroscopic experiments, one and two dimensional (1D- and 2D)-NMR and electrospray ionization MS, and comparison with the reported data led to identification of the known compounds as 3-(2′-hydroxy-n-pentyl)-1,6,8-trihydroxy-9,10-anthraquinone (3), 3-
propyl-1,6,8-trihydroxy-9,10-anthraquinone (4),\(^6,8\) 3-(trans-prop-l′-enyl)-1,6,8-trihydroxy-9,10-anthraquinone (5),\(^9\) 3-(1′-hydroxypropyl)-1,4,6,8-tetrahydroxy-9,10-anthraquinone (6),\(^5\) and 3-(1′-hydroxypropyl)-1,6,8-trihydroxy-9,10-anthraquinone (7).\(^7,8\) Compound 5 was previously obtained as a synthetic by-product.\(^9\) But this is the first report of 5 from natural sources and its completed \(^1\)H- and \(^13\)C-NMR data (see Table S1, Supplementary Materials).

Capillasterquinone A (1) was obtained as a red powder. Its high resolution quadrupole time-of-flight (HR-QTOF)-MS exhibited quasi-molecular ion peaks at \(m/z\) 341.0998 [M+H]\(^+\) and 363.0818 [M+Na]\(^+\), consistent with a molecular formula of C\(_{19}\)H\(_{16}\)O\(_6\). The NMR features indicated an anthraquinone, one main constituent reported from crinoids.\(^1\) The \(^1\)H-NMR spectrum exhibited signals of four meta-coupled aromatic protons [\(\delta_H\) 7.17 (H-2), 7.51 (H-4), 7.10 (H-5), and 6.54 (H-7), each 1H, br s], three methylenes [\(\delta_H\) 3.96 (2H, s, H-1′), 2.54 (2H, t, \(J = 7.5\) Hz, H-3′), and 1.52 (2H, m, H-4′)], and one primary methyl [\(\delta_H\) 0.86 (3H, t, \(J = 7.5\) Hz, H-5′)]. In addition, the \(^13\)C-NMR and heteronuclear single quantum coherence spectroscopy (HSQC) spectra revealed presence of 19 carbon atoms including four aromatic methines, three methylenes, one methyl, and 11 quaternary carbons [with three ketones at \(\delta_C\): 189.1 (C-9), 181.6 (C-10), and 206.7 (C-2′)]. The \(^1\)H- and \(^13\)C-NMR data of 1 were similar to those of 4,\(^8\) except for difference in the signals of the side chains. The heteronuclear multiple bond correlation (HMBC) cross-peaks of H-4 (\(\delta_H\) 7.51) with C-10 (\(\delta_C\) 181.6), H-1′ (\(\delta_H\) 3.96) with C-2 (\(\delta_C\) 125.2), C-3 (\(\delta_C\) 144.7), C-4 (\(\delta_C\) 121.0), and C-2′ (\(\delta_C\) 206.7), H-3′ (\(\delta_H\) 2.54) and H-4′ (\(\delta_H\) 1.52) with C-2′ (\(\delta_C\) 206.7), and those of H-5′ (\(\delta_H\) 0.86) with C-3′ (\(\delta_C\) 43.9) and C-4′ (\(\delta_C\) 16.5) clearly confirmed locations of the side chain at C-3 and a ketone at C-2′. Detailed analysis of other HMBC correlations (Fig. 2) led to elucidation of 1 as 3-(2′-one-n-pentyl)-1,6,8-trihydroxy-9,10-anthraquinone.
The $^1$H- and $^{13}$C-NMR data of capillasterquinone B (2) were also similar to those of 4,8 except for the presence of signals of three aromatic methines [$\delta^\mathrm{C} 128.4$ (C-2), 108.5 (C-5), and 108.2 (C-7)/$\delta^\mathrm{H} 7.25$ (1H, s, H-2), 7.18 (1H, br s, H-5), and 6.60 (1H, br s, H-7)] and one quaternary oxygenated aromatic carbon [$\delta^\mathrm{C} 156.4$ (C-4)] in 2 instead of four aromatic methines in 4. This was also confirmed by HR-QTOF-MS containing a quasi-molecular ion peak at $m/z$ 315.0863 [M+H]$^+$, consistent with a molecular formula of C$_{17}$H$_{14}$O$_6$. The cross-peaks of H-1’ ($\delta^\mathrm{H} 2.64$) with C-2 ($\delta^\mathrm{C} 128.4$), C-3 ($\delta^\mathrm{C} 143.5$), and C-4 ($\delta^\mathrm{C} 156.4$) as well as a far $^2J$ correlation of H-2 ($\delta^\mathrm{H} 7.25$) with C-9 ($\delta^\mathrm{C} 187.7$) but no $^3J$ correlation of H-2 with C-10 ($\delta^\mathrm{C} 186.4$) were observed in the HMBC spectrum of 2 indicating position of the additional hydroxy group at C-4. Detailed analysis of other HMBC cross-peaks (Fig. 2) clearly elucidated the structure of 2 as 3-propyl-1,4,6,8-tetrahydroxy-9,10-anthraquinone.

The $^1$H- and $^{13}$C-NMR data of capillasterolide (8) were essentially identical to those of 13-(2-hydroxy-3,4-dimethyl-5-oxo-2,5-dihydrofuran-2-yl) tridecanoic acid methyl ester.10 However, analysis of the integrated values for the overlapped methylene proton signal at $\delta^\mathrm{H} 1.31$ (12H, br s, H-7 to H-12) suggested that length of the saturated chain of 8 is shorter than that of 13-(2-hydroxy-3,4-dimethyl-5-oxo-2,5-dihydrofuran-2-yl) tridecanoic acid methyl ester by two methylene groups. This suggestion was confirmed by HR-QTOF-MS with ion peaks at $m/z$ 349.1991 [M+Na]$^+$ and 675.4079 [2M+Na]$^+$, corresponding with a molecular formula of C$_{18}$H$_{30}$O$_5$. The configuration at C-4 of 8 was assigned to be the same as that of 13-(2-hydroxy-3,4-dimethyl-5-oxo-2,5-dihydrofuran-2-yl) tridecanoic acid methyl ester on the basis of their similar positive optical rotations.10 Consequently, the structure of 8 was elucidated as 11-(2-hydroxy-3,4-dimethyl-5-oxo-2,5-dihydrofuran-2-yl) undecanoic acid methyl ester.
The inhibitory effect on LPS-induced NO production in RAW264.7 cells of compounds 1−8 was evaluated following the previously described protocols.11−13) Among isolates, capillasterquinone A (1) showed strong NO production inhibitory activity with an IC$_{50}$ of 5.89 ± 0.11 µM, relative to that of the positive control cardamonin$^{14)}$ (IC$_{50}$ of 2.59 ± 0.18 µM). Compounds 2−5 and 7 showed significant activity with IC$_{50}$ values of 12.02 ± 0.45, 20.89 ± 0.76, 13.18 ± 0.15, 19.05 ± 0.32, and 16.98 ± 0.98 µM, respectively, whereas 6 and 8 show less effect (IC$_{50}$ > 100 µM). The cell viability assay indicated that these compounds had little or no cytotoxic effect on the cells (data not shown). With strong inhibitory effect on NO production in macrophage RAW264.7 cells, compound 1 was further investigated on the LPS-induced iNOS and COX-2 expression. RAW264.7 cells were stimulated with 1 µg/mL LPS for 24 h in the increasing concentrations of 1. The expression levels of iNOS and COX-2 were determined by comparing with that of the loading control, tubulin, in an immunoblot analysis.$^{15}$) As a result, compound 1 reduced the LPS-induced iNOS and COX-2 expressions in a dose-dependent manner whereas the expression level of tubulin was not changed (Fig. 3). iNOS and COX-2 are known as two major inflammatory mediators. The increased production of cellular NO by iNOS is found in many chronic inflammatory and autoimmune diseases.$^{16)}$ Our results indicated that compound 1 strongly inhibited the NO production and suppressed the expressions of iNOS and COX-2 proteins. Therefore, 1 could be considered as a potential candidate for anti-inflammatory agent.

Experimental

**General experimental procedures** Optical rotations were determined on a JASCO P-2000 polarimeter (Tokyo, Japan). High resolution mass spectra were recorded on an Agilent 6530 Accurate-Mass spectrometer (CA, USA). The $^1$H-NMR (500 MHz) and $^{13}$C-NMR (125 MHz) spectra were recorded on a Bruker AVANCE III HD 500 (MA, USA) FT-NMR spectrometer.
with tetramethylsilane (TMS) was used as an internal standard. Medium pressure liquid chromatography (MPLC) was carried out on a Biotage - Isolera One system (SE-751 03 Uppsala, Sweden). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck, Darmstadt, Germany) and YMC*GEL (ODS-A, 12 nm S-150 mm, YMC Co., Ltd., Japan) resins. HPLC purification was carried out on an Agilent 1200 series preparative system equipped with a diode array detector (CA, USA). TLC used pre-coated silica gel 60 F254 (1.05554.0001, Merck) and RP-18 F254S plates (1.15685.0001, Merck), and compounds were visualized by spraying with aqueous 10% H2SO4 and heating for 3–5 min.

**Biological Material** The samples of *C. multiradiatus* (Linnaeus, 1758) were collected at Son Tra, Da Nang, Vietnam, in August 2016, and identified by Prof. Do Cong Thung, Institute of Marine Environment and Resources, VAST. The voucher specimens (ST-HB-03) were deposited at the Institute of Marine Environment and Resources and Institute of Marine Biochemistry, VAST, Vietnam.

**Extraction and Isolation** The dried samples (1.5 kg) of the crinoid *Capillaster multiradiatus* were powered and extracted with MeOH under ultrasonic condition (3 time, 1h each). The resulted solutions were filtered, combined, and evaporated under reduced pressure to obtain the methanol residue (M, 100 g). This was suspended in distilled water and partitioned in turn with *n*-hexane and CH2Cl2 to obtain *n*-hexane (H, 25.0 g) and CH2Cl2 (D, 20.0 g) extracts after removal of the solvent under reduced pressure and water layer (W). Extract D (20 g) was separated on Silica gel MPLC using the mobile phase of *n*-hexane–acetone (gradient 50:1→1:1, v/v) to obtain seven fractions, D1–D7. Fraction D3 (2.5 g) was separated by Silica gel CC eluting with *n*-hexane–acetone (8:1, v/v) to give three subfractions, D3A–D3C. Subfraction D3B (230 mg) was further separated by Silica gel CC with *n*-hexane–EtOAc (4:1, v/v), followed by YMC CC with acetone–H2O (2.5:1, v/v) to obtain compounds 2 (1.7 mg)
and 4 (14 mg). Fraction D4 (1.5 g) was further separated on YMC CC eluting with MeOH–H₂O (1:1, v/v) to give five subfractions, D4A–D4E. Purification of subfraction D4A (16 mg) on YMC CC with acetone–H₂O (2.5:1, v/v), and then on Silica gel CC with n-hexane–EtOAc (5:1, v/v) furnished compound 5 (2.3 mg). Subfraction D4B (55 mg) was separated into two smaller fractions, D4B1 (27 mg) and D4B2 (25 mg), by Silica gel CC using n-hexane-EtOAc (5:1, v/v) as eluent. Purification of fraction D4B1 (27 mg) by HPLC (Column: Cosmosil 5C18-MS-II column, 250×4.6 mm, 5 µm; eluent: ACN–H₂O 60:40; flow rate: 0.6 ml/min) obtained compounds 1 (11.0 mg) and 8 (2.1 mg). Subfraction D4C (45 mg) furnished compound 6 (2.0 mg) after subjecting it on YMC CC with eluent of acetone–H₂O (2:1, v/v). Subfraction D4D (600 mg) was separated into three smaller fractions (D4D1–D4D3) by YMC CC eluting with acetone–H₂O (1.5:1, v/v). Fraction D4D1 (30 mg) was purified by Silica gel CC with n-hexane–EtOAc (2:1, v/v) to obtain compound 3 (5.0 mg). Finally, compound 7 (4.0 mg) was purified from fraction D4E (320 mg) after subjecting it on YMC CC with acetone–H₂O (1.5:1, v/v), followed by Silica gel CC with n-hexane–EtOAc (2:1, v/v).

Capillasterquinone A (1): red powder, ¹H-NMR (DMSO-d₆, 500 MHz) and ¹³C-NMR (DMSO-d₆, 125 MHz) are given in Table 1; HR-QTOF-MS m/z 341.0998 [M+H]⁺ (Calcd. for C₁₉H₁₇O₆⁺, 341.1014) and 363.0818 [M+Na]⁺ (Calcd. for C₁₉H₁₆NaO₆⁺, 363.0839).

Capillasterquinone B (2): red powder, ¹H-NMR (DMSO-d₆, 500 MHz) and ¹³C-NMR (DMSO-d₆, 125 MHz) are given in Table 1; HR-QTOF-MS m/z 315.0863 [M+H]⁺ (Calcd for C₁₇H₁₅O₆⁺, 315.0863).

Capillasterolide (8): colorless oil, [α]D²⁵ + 8.5 (c, 0.05, MeOH); ¹H-NMR (CD₃OD, 500 MHz): δH 1.77 (1H, m, H₅-5), 1.97 (1H, m, H₅-5), 1.15 (1H, m, H₆-6), 1.26 (1H, m, H₆-6), 1.31 (12H, br s, H-7 to H-12), 1.62 (2H, m, H-13), 2.33 (2H, t, J = 7.5, Hz, H-14), 1.80 (3H,
d, J = 1.0 Hz, H-16), 1.95 (3H, d, J = 1.0 Hz, H-17), and 3.67 (3H, s, OCH3); 13C-NMR (CD3OD, 125 MHz): δC 174.5 (C, C-1), 160.4 (C, C-2), 125.7 (C, C-3), 109.2 (C, C-4), 36.9 (CH2, C-5), 24.1 (CH2, C-6), 30.5, 30.4, 30.3, 30.1, (CH2, C-7 to C-12), 26.0 (CH2, C-13), 34.8 (CH2, C-14), 176.0 (C, C-15), 8.2 (CH3, C-16), 10.8 (CH3, C-17), and 51.9 (OCH3); HR-QTOF-MS m/z 349.1991 [M+Na]+ (calcd for C18H30NaO5+, 349.1985) and 675.4079 [2M+Na]+ (Calcd for C36H60NaO10+, 675.4079).

Acknowledgements This research is funded by a grant from Vietnam Academy of Science and Technology (code: VAST.TD.DLB.03/16-18). The authors are grateful to the Institute of Chemistry, VAST for measurement of the NMR spectra and Dr. Bui Huu Tai, Institute of Marine Biochemistry, VAST for measurement of the HR-QTOF mass spectra.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials, 1D- and 2D-NMR spectra for the new compounds 1, 2, and 8 as well as 1H- and 13C-NMR data of compounds 3–7.
References


Table 1. $^1$H- (DMSO-$d_6$, 500 MHz) and $^{13}$C-NMR (DMSO-$d_6$, 125 MHz) Spectroscopic Data of 1 and 2

<table>
<thead>
<tr>
<th>Pos.</th>
<th>$\delta_C$</th>
<th>$\delta_H$ mult. (J in Hz)</th>
<th>$\delta_C$</th>
<th>$\delta_H$ mult. (J in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>161.1</td>
<td>-</td>
<td>156.1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>125.2</td>
<td>7.17 br s</td>
<td>128.4</td>
<td>7.25 s</td>
</tr>
<tr>
<td>3</td>
<td>144.7</td>
<td>-</td>
<td>143.5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>121.0</td>
<td>7.51 br s</td>
<td>156.4</td>
<td>-</td>
</tr>
<tr>
<td>4a</td>
<td>132.7</td>
<td>-</td>
<td>111.8</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>109.6</td>
<td>7.10 br s</td>
<td>108.5</td>
<td>7.18 br s</td>
</tr>
<tr>
<td>6</td>
<td>166.1</td>
<td>-</td>
<td>165.8</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>107.9</td>
<td>6.54 br s</td>
<td>108.2</td>
<td>6.60 br s</td>
</tr>
<tr>
<td>8</td>
<td>164.6</td>
<td>-</td>
<td>164.5</td>
<td>-</td>
</tr>
<tr>
<td>8a</td>
<td>108.5</td>
<td>-</td>
<td>109.0</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>189.1</td>
<td>-</td>
<td>187.7</td>
<td>-</td>
</tr>
<tr>
<td>9a</td>
<td>114.2</td>
<td>-</td>
<td>110.3</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>181.6</td>
<td>-</td>
<td>186.4</td>
<td>-</td>
</tr>
<tr>
<td>10a</td>
<td>135.1</td>
<td>-</td>
<td>134.9</td>
<td>-</td>
</tr>
<tr>
<td>1’</td>
<td>48.3</td>
<td>3.96 s</td>
<td>31.1</td>
<td>2.64 br t (7.5)</td>
</tr>
<tr>
<td>2’</td>
<td>206.7</td>
<td>-</td>
<td>21.4</td>
<td>1.63 m</td>
</tr>
<tr>
<td>3’</td>
<td>43.9</td>
<td>2.54 t (7.5)</td>
<td>13.7</td>
<td>0.94 t (7.5)</td>
</tr>
<tr>
<td>4’</td>
<td>16.5</td>
<td>1.52 m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5’</td>
<td>13.5</td>
<td>0.86 t (7.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Assignments were confirmed by HSQC and HMBC experiments
Fig. 1. Structures of Compounds 1–8.
Fig. 2. Key HMBC Correlations of Compounds 1 and 2.
Fig. 3. Inhibition of LPS-induced iNOS and COX-2 Expressions in RAW264.7 Cells by Compound 1